UNIVERSITÄT
BERN

Graduate School for Cellular and Biomedical Sciences
University of Bern

Endocrine and cellular mechanisms of decidualization in the early pregnant canine uterus: remodeling processes and role of PGE2

PhD Thesis submitted by

Felix R. M. Graubner
from **Germany**

for the degree of
Doctor of Veterinary Medicine and Philosophy (DVM, PhD)

Supervisor
Prof. Dr. Mariusz P. Kowalewski, PhD
Institute of Veterinary Anatomy
Vetsuisse Faculty, University of Zurich

Co-advisor
Prof. Dr. Susanne Ulbrich
Institute of Agricultural Sciences, Animal Physiology
Swiss Federal Institute of Technology (ETH) Zurich

Accepted by the Faculty of Medicine, the Faculty of Science and the Vetsuisse Faculty of the University of Bern at the request of the Graduate School for Cellular and Biomedical Sciences

Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

Contents

Abbreviation

αSMA = α smooth muscle actin
AAc = arachidonic acid
Ac = adenylate cyclase
AIF1 = allograft inflammatory factor 1
BSA = bovine serum albumine
cAMP/PKA pathway = cyclic AMP / protein kinase A pathway
CBR1 = carbonyl reductase 1
CD = cluster of differentiation
CL = corpus luteum
COL1 = collagen 1
COL3 = collagen 3
COL4 = collagen 4
COX2/PTGS2 = cyclooxygenase 2
CRP-LI = C-reactive protein-like
Cx26 = connexin 26
Cx43 = connexin 43
CXCL16 = chemokine ligand 16
CXCR6 = chemokine receptor 6
CXCR7 = chemokine receptor 7
CYP26A1 = retinoic acid-metabolizing cytochrome
dbcAMP = dibutyryl cyclic-AMP sodium salt
DIAPH3 = diaphanous-related formin 3
DUS = dog uterine stroma
E2 = estradiol
ECM = extracellular matrix
ECM 1 = extracellular matrix protein 1
EP1/PTGER1 = receptor 1 of PGE2
EP2/PTGER2 = receptor 2 of PGE2
EP3/PTGER3 = receptor 3 of PGE2
EP4/PTGER4 = receptor 4 of PGE2
ESR2 = estrogen receptor
FDR = false discovery rate
FN1 = fibronectin 1
IDO = idolamine 2,3-dioxygenase 1
IFN τ = interferon τ
IGF1 = insulin-like growth factor1
IGF1R = insulin-like growth factor1 receptor
IGF2 = insulin-like growth factor 2
IL = interleukin
IPO9 = importin 9
ITG = integrin
ITS = insulin-transferrin-selenium
ITIH4 = inter-alpha-trypsin inhibitor heavy chain family member 4
IVF = in vitro fertilization
LAMA2 = laminin alpha 2

Abbreviation

LH = luteinizing hormone
LXR = liver X receptor
MKi-67 = marker of proliferation Ki-67
MMP = matrix metalloproteinase
MMP2 = matrix metalloproteinase2
MMP9 = matrix metalloproteinase9
MRP: maternal recognition of pregnancy
NK cells = natural killer cells
NOV = nephroblastoma overexpressed
OXT = oxytocin
OXTR = oxytocin receptor
p = passage(s)
P = P-value
P4 = progesterone
PAPPA = pappalysin 2
PENK = proenkephalin
PG = prostaglandin
PGD2 = prostaglandin D2
PGE2 = prostaglandin E2
PGE2M = metabolite of PGE2
PGF2α = prostaglandin F2α
PGFS/AKR1C3 = synthase of PGF2α
PGH2 = prostaglandin H2
PGI2α = prostaglandin I2α
PGR = progesterone receptor
PRL = prolactin
PRLR = prolactin receptor
pSV40Tag = plasmid vector carrying the large T-antigen (Tag) of simian vacuolating virus 40
PTGDR = prostaglandin D2 receptor
PTGES = synthase of PGE2
PTGFR/FP = PGF2α receptor
RLN = relaxin
RXFP1 = relaxin receptor 1
RXFP2 = relaxin receptor 2
SIPS = subinvolution of placental sites
Tag = T-antigen
TIMP2 = tissue inhibitor of matrix proteases 2
TIMP4 = tissue inhibitor of matrix proteases 4
TNFα = tumor necrosis factor α
TxA2 = Thromboxane A2
VEGF = vascular endothelial growth factor
VEGFR2 = receptor 2 of vascular endothelial growth factor

Abstract

In the domestic dog (*Canis lupus familiaris*) little is known about the reproductive system and its regulation during gestation. In particular, the establishment and maintenance of canine pregnancy is not well understood. Therefore, descriptive *in vivo* and experimental *in vitro* studies were used in this PhD work to investigate endocrine and cellular mechanisms regulating decidualization and thereby contributing to the successful onset of canine pregnancy. The descriptive part utilized a microarray approach to determine global embryo-induced changes in the canine uterus before implantation (days 10-12 of pregnancy). The strongest up-regulated functional terms of the microarray-derived datasets indicated an embryo-induced modification of the uterine extracellular matrix (ECM). Thus, its composition and factors possibly involved in the modulation of canine uterine and utero-placental ECM were investigated in more detail at selected stages of canine gestation and at pre-partum luteolysis, involving mRNA and protein expression analyses. While only slight morphological ECM adjustments were identified at the early (i.e., pre-attachment) stage of pregnancy, following implantation intense ECM modifications were found. In particular, a decrease of major structural collagens (COL) (COL1 and -3) and laminin α (LAMA) 2 at utero-placental sites, concomitant with a gradual increase in modulators of the extracellular matrix such as tissue inhibitors of metalloproteinases (TIMPs), were prominent. The functional relevance of this loosening of uterine structural integrity and increase in its compliance has to be evaluated depending on the gestational age. Thus, whereas the decrease of COLs (e.g., COL1, -3), and LAMA2 at the beginning of pregnancy appears to be associated with trophoblast invasion and establishment of pregnancy, their further decrease observed at pre-partum luteolysis may be associated with preparation for release of fetal membranes and fetal expulsion. Although ECM degradation seems mandatory for proper trophoblast invasion, deep uterine compartments need to be protected from excessive matrix metalloproteinase activity. The detected spatio-temporal expression of tissue inhibitors of metalloproteinases (TIMP) 2 and -4 provides the first implication of their involvement in this balancing system in the dog.

The other functional terms strongly represented in the early pregnant canine uterus related to inflammatory response, regulation of cell motion and migration and angiogenesis. In particular, the cluster “inflammatory response” was of interest because also in other species immunomodulation was shown to be essential for healthy pregnancy. The microarray data were validated by assessing the expression of potential target genes related to the immune system, such as idolamine (IDO), allograft inflammatory factor (AIF) 1, chemokine ligand (CXCL) 16 and its receptor CXCR6, and CXCR7. Additionally, the microarray identified top differentially expressed genes (top scored genes) during early pregnancy. These were represented by importin 9 (IPO9), inter-alpha-trypsin inhibitor heavy chain family member 4 (ITIH) 4 and nephroblastoma overexpressed (NOV), implying their involvement in the biochemical preparation for decidualization and implantation.

Abstract

The canine microarray data were additionally compared with similar data obtained during the pre-implantation period in some other mammals displaying different placentation types, including the less invasive epitheliochorial placentation of cattle, horses and pigs, and the more invasive hemochorial placentation of humans. The data set from humans was derived from the window of implantation (WOI; period of endometrial receptivity). Interestingly, when compared with dogs, the highest similarities were found for humans vs. other species. Therefore, the dog might function as a suitable animal model for early decidualization and placentation studies in humans. Another key finding came from the comparison of canine and porcine gene sets, indicating that different mammals may utilize opposite strategies within similar regulatory pathways to ensure the successful outcome of pregnancy.

The *in vitro* study of this PhD work focused on molecular investigations of canine decidualization. For this, an immortalized dog uterine stromal (DUS) cell line was established and characterized. DUS cells maintained their decidualization capabilities when exposed to our standard, dbcAMP-induced, *in vitro* decidualization protocol. Furthermore, treatment with PGE2, a well-known cAMP inducer, demonstrated its basic capability to enhance the expression of biochemical markers of canine decidualization such as prolactin receptor (PRLR), progesterone receptor (PGR), the synthase of PGE2 (PTGES) and the respective PGE2 receptor, EP4/PTGER4. Consequently, and in agreement with our previously published observations about the presence of the PGE2-system in canine free-floating embryos, uterus and utero-placental compartments, PGE2 seems to be an important factor for canine decidualization. Finally, in a preliminary follow-up study, in order to distinguish effects mediated through particular PGE2 receptors, the functional blocking of one of the PGE2 receptors, EP2/PTGER2, has been assessed, substantiating the importance of this receptor for canine species-specific decidualization. Translating the presented results into the *in vivo* canine reproductive system, it is suggested that embryonal and uterine PGE2 are involved in regulating canine reproduction by exerting modulatory effects on the expression of PGR and PRLR.

In summary, by providing both *in vivo* and *in vitro* observations, this PhD work has helped to improve our knowledge about the canine reproductive system. In particular, the species-specific decidualization process initiated at the beginning of canine pregnancy has been characterized in more detail. Taking into consideration the importance of decidual cells for the maintenance and termination of canine pregnancy, the findings presented here seem to be also of the utmost clinical relevance.

Introduction

Embryo-maternal crosstalk, general aspects and canine species-specific regulatory mechanisms

The establishment, maintenance and termination of pregnancy in mammals are inter-connected by a complex bidirectional embryo-maternal crosstalk between the uterus and the conceptus. This crosstalk is of particular interest because miscommunication at any time of pregnancy can lead to complications and adverse effects, even resulting in fetal death.

In early pregnancy, several communication-based checkpoints need to be passed for the establishment of healthy pregnancy. These points include rescue of corpus luteum (CL) function, synchronization of the embryo and uterus in preparation for implantation, and immunomodulation (reviewed in [1]). Across all mammals, clearly, one of the most critical events needed for successful pregnancy is the extension of CL function. In this context, R.V. Short defined the original term "maternal recognition of pregnancy" (MRP) as "prevention of luteolysis" [2]. This definition appears to be correct for numerous mammals, in which the lifetime of the cyclic CL needs to be prolonged by transmission of embryo-derived signals to the uterus. These signals preserve high levels of circulating progesterone (P4) needed for maintenance of pregnancy. Thus, in ruminants, it is well known that MRP signaling is promoted by interferon (IFN) τ [3, 4], secreted by trophectoderm [5]. In pigs, MRP signaling consists of conceptus-derived estrogens [6], which redirect luteolytic prostaglandin (PG) F2α secretion, promoting its exocrine (into the uterine lumen), instead of endocrine secretion [7]. In mares, the exact mechanisms or factors involved in MRP, leading to inhibition of luteolysis, remain largely unknown. The molecular mechanisms involve, however, local, i.e., uterine, suppression of cyclooxygenase 2 (COX2/PTGS2) synthesis [8-10]. The original definition of MRP launched by R.V. Short fits the reproductive physiology of livestock. It does not apply, however, to the situation observed, e.g., in marsupials and some other eutherians (placental mammals), such as the dog, in which embryonic signaling is not required for CL maintenance to ensure successful pregnancy. Therefore, e.g., in the dog, in which the maintenance of pregnancy fully depends on the provision of luteal P4 [11], the embryo-maternal crosstalk has been defined as a functional relationship between the uterus, CL and the embryo itself [12].

Indeed, the canine reproductive system features unique species-specific mechanisms differentiating it from other domestic animals. Thus, as well as the circulating P4 concentrations fully originating from the CL of both pregnant and non-pregnant bitches [11], P4 profiles are also nearly identical in both situations [13]. Consequently, P4 is not an adequate marker of pregnancy in the dog. Only shortly before parturition does a disparity in P4 profiles become obvious, associated with a steep P4 decrease observed in pregnant animals during prepartum luteolysis [11]. In contrast, the CL of non-pregnant cyclic bitches undergoes

greatly extended luteal regression, which is accompanied by a slow P4 decrease, frequently going beyond the time point of the prepartum luteolysis (reviewed in [14]). In the literature, the phase of luteal activity in bitches is referred to as diestrus [12, 15].
Similar to P4, estrogens present secretion profiles that are similar in pregnant and non-pregnant bitches [16, 17]. Furthermore, in the dog, in contrast to livestock, there is no pregnancy- or parturition-related increase of estradiol (E2) [16, 17]. Interestingly, however, in contrast to P4 or E2, placental relaxin (RLN) increases greatly in the maternal circulation during pregnancy and is the only marker of pregnancy known so far for the dog [13]. It appears in the peripheral blood shortly following implantation, i.e., around day 25 of gestation, and is of fetal placental origin [18]. Whereas RLN is synthesized primarily in the cytotrophoblast, its respective receptors, RXFP1 and RXFP2, are also strongly present in placental endothelial cells and in decidual cells, respectively [18]. Although RLN is nearly absent from the maternal circulation during the first weeks of pregnancy [13], nevertheless, local uterine effects cannot be excluded [18].

Despite the similar profiles of P4 and estrogens in non-pregnant and early (pre-attachment) pregnant bitches, it seems obvious that in order to prevent rejection of embryos, there must be a species-specific embryo-maternal communication, although it is not related to the avoidance of luteolysis. Yet, little is known about the basic mechanisms regulating canine pregnancy, in particular during early gestational stages, which could be one of the reasons why *in vitro* fertilization (IVF) in dogs is unsuccessful. This also accounts for the differences in uterine physiology between non-pregnant and pregnant bitches.
Some earlier studies addressed the uterine biochemical milieu with regard to factors responsible for proper embryo-maternal interaction prior to and at the time of implantation. These factors represented, e.g., the vasoactive and angiogenic families, and the immune system, as well as factors involved in cell-to-cell communication.
Thus, modification of the canine uterine angiogenic system is, among other features, characterized by significant up-regulation of the uterine vascular endothelial growth factor (VEGF) -A family (i.e., VEGF 164, -182, and -188) as well as one of the respective receptors, VEGFR2 [19, 20], during early (pre-attachment) pregnancy. In this regard, it seems that the provision of a sufficient blood supply in preparation for implantation is conserved across mammals because also in other placentals (eutherians), such as humans, angiogenic modifications are observed in early pregnancy (reviewed in [21]). Interestingly, mirroring the complexity of early embryo-maternal communication, the angiogenic system is connected to the processes of decidualization [21] and immunomodulation [22, 23], which are other mandatory steps in the establishment of successful pregnancy in humans. It is, however, not clear if similar correlations between the respective processes also apply in the dog.

With the goal of characterizing early (pre-attachment) pregnancy markers in the dog, previous studies investigated possible involvement of the immune system and immune-reactive proteins, like acute phase proteins. Thus, in peripheral blood, the concentration of some acute

phase proteins was reported to increase during early pregnancy. This includes, e.g., fibrinogen and serum C-reactive protein-like (CRP-LI), which were elevated 21 to 30 days after the LH (luteinizing hormone) surge [24, 25]. However, these proteins act as mediators of inflammation and could, therefore, be increased in dogs suffering from infection or trauma. Consequently, elevated blood concentrations of these proteins cannot be used as a reliable marker of canine pregnancy.

More recently, the spatio-temporal uterine expression of several cytokines and immune cell receptors has been investigated. Cluster of differentiation (CD) 4, interleukin (IL) 6 and tumor necrosis factor (TNF) α seemed to be predominantly linked to the non-pregnant uterus [26, 27]. Conversely, suggesting their involvement in the establishment of a healthy pregnancy, IL4, CD8 and interferon (IFN) γ seemed to be mostly associated with early (pre-attachment) pregnancy [26, 27]. It is noteworthy that the importance of uterine immunomodulation in the establishment of pregnancy is further underlined by the fact that, e.g., in humans, impaired uterine immunomodulation can lead to preeclampsia (reviewed in [28]).

In view of the complexity of immunomodulation at the beginning of pregnancy in other species, it seems clear that the involvement of immune system-derived factors in modulating embryo-maternal communication in the dog is not yet well characterized and requires further research.

Lately, in addition to embryo-induced alterations of the immune system, embryo-triggered uterine mitogenic/proliferative and tissue remodeling effects have been addressed [19, 26, 29]. These effects are implied by the significant modulation of insulin-like growth factor (IGF) 2 [29], some of the integrins (ITG) (e.g., ITGA2B, ITGB2 and ITGB3) [19] and matrix metalloproteinase (MMP) 2 [26] expression, when comparing uteri of non-pregnant and early pregnant (pre-attachment) dogs. In more detail, these remodeling effects are elaborated in one of the following chapters, *"ECM remodeling during establishment of pregnancy"*. It is also worth mentioning that some of these modifications are also linked to the species-specific decidualization process (see also chapter *"Decidualization"*).

Moreover, canine free-floating embryos were analyzed for the expression of specific genes possibly involved in the embryo-maternal crosstalk [26, 29, 30]. Genes detected in free-floating blastocysts indicate possible interactions with the uterus through the prostaglandin and immune systems as well as their involvement in the modulation of growth and invasion [26, 29, 30].

In summary, there is only limited information available about the embryo-maternal crosstalk in the domestic dog. In particular, information about uterine modifications at the early (pre-attachment) stage of pregnancy is scarce, and only a few canine uterine genes have been closely characterized. Obviously, deeper knowledge about functional systems involved in this bi-directional crosstalk is still needed. Therefore, applying a more comprehensive approach, such as microarray analysis, would help to provide better understanding of this very complex interplay. In particular, embryo-maternal communication at the beginning of canine pregnancy, prior to the attachment of embryos, seems a worthy target since, being devoid of

an antiluteolytic signal, the dog is indeed a unique species among mammals. Moreover, IVF in dogs is unsuccessful due to perturbed embryo development and implantation failure. In view of dogs as both important pets and laboratory animals, there is an urgent need to enhance our knowledge in this field.
Given the commonality of reproductive pathologies of dogs and humans, it appears that reproductive events in these species are closely related. Thus, a fundamental understanding of the canine reproductive system might provide useful information for human reproductive research. The translational aspects need, therefore, to be strongly emphasized. However, uterine changes prior to implantation have not been compared between these two species.

Decidualization

Successful implantation is dependent on proper synchronization of the embryo and uterus. In some species, in particular those exhibiting invasive types of placentation, as a consequence of this synchronization process, uterine decidual tissue is formed. The term *decidua* derives from the respective Latin verb *decider*, with the meaning to die, to fall off, or to detach. Its origin is in describing the fate of the human decidua, which in response to falling circulating P4 levels, is shed during menstruation [31, 32]. It has also been adopted for describing the deciduate type of placentation, and relates to mammals that develop more intimate placental feto-maternal interaction and shed maternal uterine tissues when expelling the placenta at birth. This kind of placentation is characteristic of endotheliochorial placentae observed in carnivores (e.g., dogs and cats [29, 33, 34]), and of the placenta hemochorialis of humans and rodents (reviewed in [35].
The formation of decidua (i.e., decidualization) is very complex and is connected to numerous events important for healthy pregnancy, such as spiral artery remodeling observed in humans [21], strong immunomodulation [36] and tissue remodeling [37-39]. The participation of decidual tissue in remodeling uterine structures is discussed in more detail in the chapter *"ECM remodeling during establishment of pregnancy"*.

Decidualization can be defined on both molecular and morphological levels. Morphologically, uterine spindle-shaped mesenchymal stromal cells transform into rounded epithelioid-like cells (so-called decidual cells) [40-42]. The first macro-anatomical reports on human decidual tissue were published in 1774 by W. Hunter [43, 44]. Further and more detailed studies revealed that this tissue is also formed in other mammals, revealing species-specific characteristics. In humans, decidualization occurs in every sexual cycle (reviewed in [35]) while in rodents and dogs, besides a suitable uterine endocrine environment, the presence of implanting blastocysts is needed for the activation of decidualization [29, 45]. Also, the morphology of decidual tissue is different among species. Thus, in species with hemochorial placentation *decidua parietalis* can be found at the luminal interplacental compartments (i.e., those parts of the uterus not involved in the formation of the placenta), while *decidua basalis* is found at placentation sites [46, 47]. Additionally, a *capsula decidualis* embeds the embryo

within the uterine lumen [46, 47]. In contrast, formation of the less invasive canine endotheliochorial placenta is associated with development of the so-called girdle placenta and species-specific decidualization [47]. Importantly, canine decidualization is exclusively present in the uterine compartments that are attached to the fetal trophoblast.

Indeed, most details about the molecular mechanisms of decidualization are known for humans and rodents. In humans, the decidualization process is triggered by hormonal changes activating the cAMP/PKA pathway (cyclic AMP/protein kinase A pathway) [41, 48, 49]. P4 is not the sole factor required for decidualization [41, 50] but is among the most important constituents of the cascade, facilitating this process by increasing intracellular levels of cAMP [41]. It initiates immune responses leading to the attraction of natural killer (NK) cells, stimulation of chemokine synthesis, and induction of quiescence of the myometrium [51, 52]. All of these events harmonize to support decidua formation. In addition, the preservation of decidual tissue is P4-dependent, because blocking the function of P4 receptors results in activation of pro-inflammatory pathways involved in menses and shedding of decidua [53]. As indicated by *in vitro* decidualization of human uterine stromal cells [49], and *in vivo* experiments with mice [54], P4 seems to be involved in increasing the expression of important factors characteristic of decidualization, such as prolactin (PRL). Surprisingly, and in contrast to other species [41, 55], an increase in PRL itself does not occur during the canine decidualization process [29]. However, the PRL receptor (PRLR) is clearly present in epithelial compartments of the canine uterus prior to implantation [56] and PRL increases following implantation [57]. Thus, PRL could contribute to decidualization also in the canine species. This appears plausible because the PRLR is also present in the canine invasive trophoblast [56].

In addition to increased uterine PRLR expression prior to trophoblast attachment at day 10-12 after fertilization, increased mRNA levels of IGF2 are observed [29]. Importantly, pointing towards species-specific regulation of the establishment of pregnancy, in the dog the uterine expression of other pregnancy related factors, such as IGF1, IGF1-receptor (IGF1R), oxytocin receptor (OXTR), P4-receptor (PGR) and estrogen receptor 2 (ESR2), although clearly detectable, does not differ between early (pre-attachment) pregnant and non-pregnant diestrous uteri [29].

Importantly, the only cells in the canine placenta carrying the nuclear PGR are the maternal decidual cells [58]. During the invasive growth of trophoblast into maternal tissues, nearly all cellular constituents of the uterus located luminalward from the connective tissue layer separating superficial from deep glands, are enzymatically removed (digested). This is due to the proteolytic activity of the trophoblast. Only two cell populations, i.e., maternal decidual and endothelial cells, can restrain the invasive properties of trophoblast [58, 59]. When the physiological function of decidual cells is blocked, e.g., by antigestagen-(PGR-blocker)-induced withdrawal of P4, abortion or preterm parturition occurs [58]. As a further consequence, similar to during natural P4 withdrawal pre-partum, the placental luteolytic cascade leading to increased output of PGF2α becomes activated [58, 60]. This prepartum

increase of prostaglandin synthesis apparently originates mostly in the fetal part of the placenta (trophoblast), where the respective biosynthetic pathways are localized [58, 60, 61]. The importance of the placental feto-maternal communication between the maternal decidual cells expressing the nuclear PGR and fetal trophoblast during initialization of the luteolytic cascade is, thus, apparent. Furthermore, the mediating role of decidual cells during the prepartum luteolytic cascade seems to be, at least in part, mediated through the OXTR, which is specifically expressed in these cells [62]. Its placental expression is strongly induced both during normal and antigestagen-mediated abortion/parturition. Yet, the underlying cell-to-cell communication pathway between fetal and maternal compartments of the canine placenta remain not fully elucidated.

A more detailed description of the luteolytic cascade is presented in the chapter *"Prostaglandins"*.

Recently, research aimed at better understanding of the canine decidualization process was initiated in our group using *in vitro* studies. First, the basic capability of primary canine uterine stromal cells to decidualize *in vitro* was demonstrated [63]. Similar as during early *in vivo* decidualization, *in vitro* decidualized cells displayed higher metabolic and secretory activities and induced expression of selected decidualization markers [63]. However, primary cell cultures are challenging and are unsuitable for consecutive studies because multiple passaging will alter their biochemical and biological properties, as indicated by up-regulation of cytokeratin (a marker of epithelial cells, which is absent in mesenchymal-derived decidual cells). Furthermore, the limited access to appropriate (in terms of cycle and health status) uterine tissues is hindering research. Therefore, *in vitro* research on canine decidualization would benefit from the establishment of an immortalized cell line, providing researchers with a reliable cellular model and opportunities to perform consecutive experiments on cells that could be maintained over long periods of time or frozen for further use.

Prostaglandins

Prostaglandins (PGs) belong to a group of hormones that are associated with physiological functions (i.e., homeostasis) and pathological mechanisms (i.e., inflammation) in mammals (reviewed in [64, 65]). PG synthesis starts with the release of arachidonic acid (AAc) from the plasma membrane by phospholipases. In a second step, AAc is metabolized by the upstream regulator COX2/PTGS2 into PGH2, which is the precursor of many PGs and prostanoids [64, 65]. Following this common pathway, specific PG-synthases prepare the different bioactive PGs (e.g., PGE2, PGF2α, PGI2α, PGD2). These eicosanoids are produced in nearly all cell types and mediate their functions through endocrine or paracrine mechanisms. While in healthy tissues PGs production and levels are generally low, during inflammation they increase dramatically, even before recruitment of leukocytes and infiltration by immune cells [64]. One exception to this general rule seems to apply to the reproductive tract where under physiological conditions, such as during decidualization, a high presence of PGs is observed

(reviewed in [66]). Moreover COX2/PTGS2-deficient mice display multiple reproductive defects, which even further demonstrates the importance of the PG-system for healthy pregnancy [67].

With regard to reproduction, one of the best-known PGs is PGF2α. It acts on the CL causing luteolysis and thereby cessation of P4 production. In the dog, active luteolysis is only observed in pregnant bitches (reviewed in [14]). As mentioned elsewhere, the source of PGF2α is the canine placenta [58, 60]. Whereas the uterine expression of COX2/PTGS2 remains unchanged until late gestation, it is strongly induced at prepartum luteolysis [58]. Surprisingly, however, the expression of the synthase of PGF2α (PGFS/AKR1C3) decreases at prepartum luteolysis following its high levels at mid-gestation [58, 60]. Belonging to the aldo-keto reductase family of enzymes, AKR1C3 is the only PGF2α-synthase known so far for the dog [60]. Its biochemical actions on the conversion of PGH2 to PGF2α have been documented [60]. Its localization at the feto-maternal contact zone suggests possible local involvement of this enzyme in maintenance of pregnancy, e.g., by modulating the invasive properties of trophoblast. A role of AKR1C3 in regulating the decidualization process in the dog also appears plausible. This would resemble the situation observed in rats, in which locally (intrauterine) administered PGF2α provokes the decidual cell reaction [68].

Another important PG is PGE2. Its biosynthetic pathway is connected with that of PGF2α not only by sharing a common upstream regulator (COX2/PTGS2), but also by the activity of carbonyl reductase 1 (CBR1) which can convert PGE2 into PGF2α [69]. However, still, the main pathway of PGE2 synthesis involves the conversion of PGH2 to PGE2 by the synthase of PGE2 (PTGES). The active PGE2 can bind to 4 different PGE2 receptors, designated as EP/PTGER 1-4. The EP1/PTGER1 is coupled to a Ca^{2+}-influx and the cAMP/PKC pathway, while EP3/PTGER3 inhibits the activation of adenylate cyclase (AC) (reviewed in [70]. The EP2/PTGER2 and EP4/PTGER4 share similar activation mechanism through the cAMP/PKA pathway (reviewed in [70]. At this point, it is noteworthy to re-emphasize that the decidualization process is mediated through the cAMP/PKA pathway [71], and that in human and rodent stromal cells PGE2 was shown to induce the decidual reaction [72-74]. Deactivation of both major PGs (PGE2 and PGF2α) occurs via prostaglandin dehydrogenase (15-HPGD) through its oxydoreductase activity converting the active enzymes to their inactive metabolites, PGEM and PGFM, respectively.

As for further roles of PGE2 in regulating reproductive events, e.g., in sheep and humans, it is involved in softening of the cervix prior to and during uterine contractions [75, 76]. Similarly, in the canine species, PGE2 appears to fulfill complex pregnancy-associated functions both in the ovary and the uterus. It is noteworthy that only one canine-specific PTGES is known so far, which has been cloned and characterized in our laboratory [58, 61]. Importantly, during the early luteal phase, locally produced PGE2 is among the important luteotrophic factors [77]. At the same time, i.e., during early diestrus in pregnant bitches, uterine expression of the

synthase of PGE2 (PTGES) and EP2/PTGER2, significantly increases from the non-pregnant stage towards early (pre-attachment) pregnancy [29]. This increase indicates the possible involvement of PGE2 in canine decidualization, similar to rodents and humans, in which PGE2 accelerates P4- and cAMP-mediated decidualization [49, 73, 74, 78]. The importance of PGE2 for healthy canine pregnancy is further underlined by the constant presence of uterine and placental PTGES, EP2/PTGER2 and EP4/PTGER4 throughout pregnancy [29, 61]. Furthermore, in addition to immunomodulatory factors, canine free-floating embryos also express factors of the PG system, including PTGES, EP2/PTGER2 and EP4/PTGER4 [29]. This implies a role of PGE2 in local functional modulation of the uterus during preparation for implantation and implies embryo-protective effects [79].

Deeper insights into the possible involvement of PGs in canine decidualization were provided by *in vitro* studies with primary canine uterine stromal cells. Thus, when submitted to *in vitro* decidualization, these cells revealed enhanced expression of key factors involved in the supply of PGs, such as COX2/PTGS2, PTGES, PGFS/AKR1C3 and the respective PG receptors [63].

The concomitant expression of PTGES, EP2/PTGER2 and EP4/PTGER4 in the canine early pregnant (pre-attachment) uterus and free-floating blastocysts gives rise to the question of possible auto- or paracrine feedback mechanisms of PGE2 at the beginning of canine pregnancy. Therefore, investigations of PGE2-mediated effects on the decidualization process in the dog seem to be a worthwhile opportunity for future studies. Subsequently, the differences between EP2/PTGER2- and EP4/PTGER4- mediated, cAMP/PKA-regulated effects on uterine gene expression in early pregnancy should be determined.

ECM remodeling during establishment of pregnancy

The endometrial extracellular matrix (ECM) is a complex component of the uterus. It is located in between the cells and its main function is to provide physical support. Additionally, it can serve as a reservoir for hormones and acts as a regulator of differentiation and cell growth [80, 81]. Due to the combined actions of matrix synthesis and degradation, the ECM is in a fluid "steady state" [82]. Its degradation is carried out by matrix metalloproteinases (MMPs), which have important functions during pregnancy. Thus, during pregnancy, the balance of the uterine ECM "steady state" is altered [83-86]. In humans, trophoblast cells secrete MMPs [87] which are needed for proper uterine invasion [88]. Some MMPs, e.g., MMP2 and MMP9, have also been found in uterine and placental tissues of dogs [89-91].

As mentioned elsewhere, the invasive placentation in dogs is accompanied by formation of decidual tissue. Interestingly, there is a bidirectional connection between decidual cells and the uterine ECM. Decidual cells change the composition of the uterine ECM by secretion of basal lamina-like material [92-94] and the uterine ECM influences trophoblast invasion [95] and remodeling of decidua [37-39]. Furthermore, the decidualization process in rodents is accompanied by additional modifications of the ECM, such as clearing hyaluronic acid from

the ECM in the uterus [96], and decreasing the most abundant structural protein, collagen (COL) 1 [97]. Also in humans, the ratio between collagen-ECM proteins and non-collagen proteins is altered during pregnancy [98, 99]. All these events indicate strong remodeling of the uterine ECM, not only in preparation for implantation but also at later stages of gestation. Ultimately, the uterus is transformed into a large organ that contains the fetus/fetuses, fetal membranes, fetal fluids and the placenta.

In view of these strong transformational processes it seems obvious that there must exist some balancing systems to protect the uterus from over-excessive remodeling. In dogs, none of these balancing systems is well known so far. It appears, however, plausible that tissue inhibitors of matrix proteases (TIMPs) might be involved in this process, as also implied by studies in other species, e.g., humans (reviewed in [100]).

In the dog, some peculiar pathological conditions may occur, such as subinvolution of placental sites (SIPS), indicating functional alterations of the balancing system. Because canine SIPS shares some similarities with human *placenta accreta*, understanding of proper and impaired ECM remodeling in dogs could be helpful for investigating placental functions in general.

In summary, uterine ECM modifications at the beginning of pregnancy occur regardless of the invasive type of placentation. Following implantation and placentation, species-specific effects are expected. In the canine species the composition of the uterine ECM has not yet been investigated in great detail. It is likely, however, that species-specific decidualization and placentation will determine uterine remodeling processes characteristic of the canine species. Importantly, the similarities between some adverse conditions observed during placentation in humans and dogs imply possible inter-species translational aspects.

Hypothesis and aims of the thesis

Based on our previous findings on endocrine and cellular mechanisms regulating canine decidualization [29, 63], here, new insights into successful onset of pregnancy are provided.

The **general hypothesis** of this PhD work is that the decidualization process in the dog (*Canis lupus familiaris)* is characterized by species-specific morpho-functional signatures. It starts prior to implantation and results in the formation of maternally-derived placental decidual cells. This process can be identified *in vivo* by the detection of molecular markers, and can be, at least in part, recapitulated using cell-culture systems.

Following this general hypothesis, this PhD work focuses on a deeper characterization of decidualization in the early pregnant (pre-attachment) canine uterus, remodeling processes and role of PGE2.

We hypothesize that canine pregnancy is characterized by:

- The early embryo-maternal cross-talk, the functional signature of decidualization which can be more closely characterized using a transcriptomic approach based on microarray analysis.
- The uterine ECM reorganization, which is initiated by free-floating embryos.
- The active participation of PGE2 as an important stimulus capable of inducing decidualization in canine uterine stromal cells *in vitro*.

The following aims have been investigated to test the proposed hypotheses:

AIM 1: Characterization of factors involved in the processes of early canine decidualization and implantation.

This has been investigated by the evaluation of microarray data obtained from early pregnant (pre-attachment) and corresponding non-pregnant canine uteri. By sorting the identified differentially expressed genes into molecular systems, the complete molecular signature of the presence of early embryos on the uterine milieu has been outlined.

Based on datasets available for other species, additional analyses focused on comparison of the canine pre-implantation uterus with uterine responses to the presence of early conceptuses in other species representing different placentation types, either less (epitheliochorial) or more (hemochorial) invasive. Consequently, comparisons were made with datasets obtained from cattle, horses and pigs, as well as with microarray data obtained from humans.

AIM 2: Analysis of canine uterine tissue remodeling at selected time points during pregnancy: pre-implantation (compared with non-pregnant controls), post-

implantation, mid-gestation and natural prepartum luteolysis. Following implantation, both interplacental uterine sites and placentation sites were taken into consideration. The main focus was on the spatio-temporal expression of different structural ECM proteins such as collagens, intercellular adhesion molecules and inhibitors of matrix metalloproteinases.

AIM 3 Establishment of a canine uterine stromal cell line, verification of its potential to decidualize *in vitro*, and characterization of PGE2-induced expression of decidualization markers.

Results

Manuscript 1: Uterine responses to early pre-attachment embryos in the domestic dog and comparisons with other domestic animal species

Graubner FR, Gram A, Kautz E, Bauersachs S, Aslan S, Agaoglu A, Boos A, Kowalewski MP

Published in: Biol Reprod. 2017 Aug 1;97(2):197-216. doi: 10.1093/biolre/iox063. PMID:28651344

Contribution:
Felix R. Graubner was involved in development of the concept of the study, experimental design, generating data, analysis and interpretation of data and writing of the manuscript.

Biology of Reproduction, 2017, 97(2), 197–216
doi:10.1093/biolre/iox063
Research Article
Advance Access Publication Date: 23 June 2017

OXFORD

Research Article

Uterine responses to early pre-attachment embryos in the domestic dog and comparisons with other domestic animal species†

Felix R. Graubner[1], Aykut Gram[1], Ewa Kautz[1], Stefan Bauersachs[2], Selim Aslan[3], Ali R. Agaoglu[4], Alois Boos[1] and Mariusz P. Kowalewski[1,*]

[1]Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland; [2]Institute of Agricultural Sciences, Animal Physiology, Swiss Federal Institute of Technology (ETH) Zurich, Zurich, Switzerland; [3]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Near East University, Nicosia, North Cyprus and [4]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey

*__Correspondence:__ Mariusz P. Kowalewski, Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland. Tel: +0041-44-6358784; Fax: +0041-44-6358943; E-mail: kowalewskipl@yahoo.de or kowalewski@vetanat.uzh.ch

†__Grant Support:__ This research is supported by The Swiss National Science Foundation (SNSF); research grant number 31003A_160251.

Received 11 April 2017; Revised 15 June 2017; Accepted 21 June 2017

Abstract

In the dog, there is no luteolysis in the absence of pregnancy. Thus, this species lacks any anti-luteolytic endocrine signal as found in other species that modulate uterine function during the critical period of pregnancy establishment. Nevertheless, in the dog an embryo-maternal communication must occur in order to prevent rejection of embryos. Based on this hypothesis, we performed microarray analysis of canine uterine samples collected during pre-attachment phase (days 10-12) and in corresponding non-pregnant controls, in order to elucidate the embryo attachment signal. An additional goal was to identify differences in uterine responses to pre-attachment embryos between dogs and other mammalian species exhibiting different reproductive patterns with regard to luteolysis, implantation, and preparation for placentation. Therefore, the canine microarray data were compared with gene sets from pigs, cattle, horses, and humans. We found 412 genes differentially regulated between the two experimental groups. The functional terms most strongly enriched in response to pre-attachment embryos related to extracellular matrix function and remodeling, and to immune and inflammatory responses. Several candidate genes were validated by semi-quantitative PCR. When compared with other species, best matches were found with human and equine counterparts. Especially for the pig, the majority of overlapping genes showed opposite expression patterns. Interestingly, 1926 genes did not pair with any of the other gene sets. Using a microarray approach, we report the uterine changes in the dog driven by the presence of embryos and compare these results with datasets from other mammalian species, finding common-, contrary-, and exclusively canine-regulated genes.

Summary Sentence

Pre-implantation embryos invoke functional changes in the canine uterus related to ongoing structural remodeling and immunological modulation; comparisons with different mammals reveal similarities and differences in maternal pregnancy recognition.

Key words: dog (*Canis lupus familiaris*), embryo-maternal communication, early pregnancy.

Introduction

The foundation of every successful pregnancy is the embryo-maternal cross-talk synchronizing blastocyst development and receptivity of the uterus in preparation for implantation. One of the master regulators in the underlying endocrine and molecular regulatory mechanisms is luteal progesterone (P4). It acts by inducing expression of multiple factors facilitating implantation, e.g., those involved in the secretion of endometrial histiotroph or exerting immunomodulatory effects. In most species, besides P4, estrogens are also needed for orchestrated expression of uterine growth factors, cytokines, and prostaglandins (PG) that work locally in an autocrine or paracrine manner [1]. In addition to embryotrophic effects, uterine fluids also contain signaling molecules of embryonic origin (reviewed in [2–4]). These embryo-derived factors prevent luteolysis and maintain the luteal lifespan beyond recurrent cyclic activity, which is a mechanism referred to as "maternal recognition of pregnancy" [5]. Thus, the bidirectional embryo-maternal cross-talk starts before the embryonic tissue becomes intimately attached to the uterine epithelium. Among species, this early pregnancy signal recognizing the presence of pre-attachment embryos varies, including the two best-known endocrine mechanisms comprised of estrogens produced by porcine trophoblast [6] or interferon tau (IFN tau) produced by early conceptuses in ruminants [7, 8]. Also, the duration of the pre-implantation period varies among the domestic animal species: e.g., the elongating conceptus adheres to the uterine epithelial surface and placentation starts around day 19 of gestation in ewes [9, 10], or around day 22 in cattle [11], compared with the implantation in mares that is delayed until around day 37 of gestation [12].

Interspecies differences in regulatory mechanisms involved in the onset and maintenance of pregnancy become even more apparent when the reproductive physiology of the domestic dog (*Canis lupus familiaris*) is taken into consideration. Thus, for example, the classical definition of maternal recognition of pregnancy does not apply to the dog, because in the absence of pregnancy in this species there is no luteolytic principle [13–16]. The dog is also the only domestic animal species devoid of placental steroids and, therefore, fully dependent on luteal P4 for successful pregnancy [17]. In this context, it is noteworthy that the canine uterus is physiologically exposed to relatively high amounts of circulating P4, starting with at least 5 ng/ml at the time of ovulation and reaching average levels of 30–35 ng/ml within the first 15-30 days of pregnancy [17]. Consequently, P4 is considered to be the predominant luteal steroid needed for the establishment of pregnancy in previously estrogenized dogs [17, 18]. Furthermore, in addition to the similar estradiol-17 beta (E2) profiles in pregnant and non-pregnant bitches [19, 20], and in contrast to livestock [21–23], there is no pregnancy- and/or parturition-associated increase in E2 observed in the dog [19].

It is obvious that, even in the absence of an anti-luteolytic principle, the canine embryo must be recognized, otherwise it would be rejected. Apparently, therefore, there must exist different regulatory mechanisms responsible for embryo maternal communication in the dog, or at least mechanisms that are not directed toward the suppression of luteolysis. Based on this assumption, recently, embryo-related changes in the canine uterus were described during the early embryonal free-floating phase (days 10-12 of gestation) [24]. First, clearly distinguishable morphological changes pointing toward the onset of pre-invasive uterine decidualization were, however, associated with the attachment of the embryo to the uterine surface (around day 17 of pregnancy) [25]. Thus, biochemical and functional changes appear to be detectable earlier than morphological ones [25]. The latter, i.e., biochemical responses of the pre-implantation canine uterus to the presence of embryos, are mirrored in the modulated expression of some genes related to the processes of implantation and placentation, e.g., *IGF2*, *PGR*, *ER alpha/ESR1*, and *PRLR* [24]. Among the members of the PG family system previously implicated in early canine embryo-maternal communication [26], uterine expression of *PGT*, *PTGES*, *PGFS/AKR1C3*, and their respective receptors, *FP (PTGFR)* and *PTGER2 (EP2)* was increased in the presence of free-floating embryos [24]. Following implantation, further considerable differences become obvious between the dog and other domestic animal species regarding invasive vs. non-invasive growth of the trophoblast, leading to the formation of the canine endotheliochorial placenta. This process is associated with strong morpho-functional remodeling of the uterine stromal cells, resulting in the development of species-specific decidual cells. The functional importance of these cells is underlined by the fact that they are the only cells of the canine placenta exhibiting the PR [27, 28].

Cumulatively, especially taking into account the species-specific lack of luteolysis, the decidualization and the invasive placentation type, the domestic dog appears to be an interesting model for investigating comparative aspects relating to different strategies of maternal recognition of pregnancy that have evolved in mammals. Therefore, here we used a transcriptomic approach by microarray analysis in order to obtain deeper insight into the possible underlying regulatory mechanisms involved in the cross-talk between the early pregnant canine uterus and pre-implantation embryos (days 10-12).

Materials and methods

Sample collection

For the present study, fourteen (n = 14) healthy, cross-bred bitches aged 2-8 years were used. Hormonal status was monitored by regular measurements of progesterone (P4) concentrations every 2-3 days starting with the proestrous bleeding and cytological evaluation of vaginal smears. The day of ovulation was determined by detecting circulating P4 levels of >5 ng/ml, and by vaginal cytology. Estimating the time needed for maturation of canine oocytes within the oviduct (on average 2-3 days after ovulation), bitches were mated/inseminated 2 days after ovulation (defined as day 0 of gestation). Uterine samples were collected through routine ovariohysterectomy at early pregnancy before the free-floating embryos became intimately attached to the uterine surface (pre-implantation group, days 10-12 of pregnancy). In eight (n = 8) bitches, early pregnancy was confirmed by recovering embryos by uterine flushing on days 10-12 of gestation (early pregnant group). Uterine samples from the remaining six (n = 6) dogs were used as negative controls (non-pregnant group). All experimental procedures were carried out in accordance with animal welfare legislation (permit no. 2008-25-124 from the Faculty of Veterinary Medicine, University of Ankara). Following surgery, uterine tissue samples (including all anatomical layers) were trimmed of surrounding connective tissues and immersed for 24 h at + 4°C in RNAlater (Ambion Biotechnologie GmbH, Wiesbaden, Germany), an aqueous reagent for stabilization of cellular RNA; prolonged storage was at -80°C. Subsequently, all uterine tissue samples from the two experimental groups (early-*pregnant and non-pregnant*) were used for semi-quantitative real-time PCR.

Four (n = 4) randomly chosen samples from each group were used for microarray analysis.

Microarray hybridization and data analysis

Total RNA was isolated from eight randomly chosen uterine samples (n = 4 per group, as indicated above). First, TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate RNA that was further purified using the PureLinkTM RNA Mini Kit (Cat. 12183018A) (Ambion Life Technologies, Reinach, Switzerland). Both applications were carried out according to the manufacturers' instructions. After RNA extraction, DNase treatment was performed following the supplier's protocol using RQ1 RNAse-free DNAse (Promega, Dübendorf, Switzerland). The purity and quantity of the RNA were determined with a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific AG, Reinach, Switzerland). The quality of total RNA was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) ensuring homogeneous distribution of high RIN (RNA integrity numbers) between samples. Microarray analysis was performed using a custom Agilent 8 × 60 k microarray. The design was based on the Agilent catalog array AMADID G2519F-021193 Canine Gene Expression Microarray, canine (Cfa) RefSeq transcripts from NCBI November 2012 and Ensembl 69 transcripts. In total, the chip covered 20′293 canine genes. Cy3-labeled cRNA was produced with the Low-Input Quick Amp Labeling Kit, one color (Agilent Technologies) according to the manufacturer's instructions. After hybridization and washing, the slides were measured using an Agilent DNA Microarray Scanner (G2505C; Agilent Technologies) at a resolution of 2 μm. The Feature Extraction Software 10.7.3.1 (Agilent Technologies) was used for image processing. Signals were filtered based on "well above background" flags (detection in three of four samples), and normalized with the Bioconductor package VSN [29]. Distance matrix and heatmap based on pairwise distances (Bioconductor package geneplotter) were used for quality control of the data. Analysis of significances was performed with the Bioconductor package Limma [30]. The "FDR"-method (False Discovery Rate; FDR 10%, i.e., adjusted P-value < 0.1) was applied for the correction of multiple testing. The P-value was adjusted to $P < 0.01$. Differentially expressed genes (DEG) were identified in the contrast (i.e., pairwise comparison) "pregnant samples" vs. "non-pregnant samples." Quantitatively enriched functional categories were identified using the "functional annotation clustering" tool of Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.8; http://david.abcc.ncifcrf.gov/) [31]. DAVID analysis was performed using information from the following databases: Gene Ontology (GO), biological process (BP), cellular component (CC), and molecular function (MF), single protein of protein information resource (SP_PIR), protein domains or sites (INTERPRO), and pathways extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Cytoscape analysis was prepared using the ClueGO plug-in in Cytoscape 3.0 [32] to visualize enriched functional networks. Ingenuity Pathway Analysis software (IPA, build version 366632M, content version 26127183, QIAGEN, Redwood City, USA) was further used to identify and visualize canonical pathways and main functional terms within the dataset.

Additionally, the whole microarray-derived canine gene set (microarray signals filtered on "well above background" flags, detection in at least three of four samples) was ranked according to a score based on log2 fold change and P-value ((2+log2(fold change))* -log10(P-value)) and used for gene set enrichment analysis (GSEA) with gene sets differentially expressed in other species (either up- or down-regulated). For each comparison, the gene set for dogs was compared separately with available lists of DEG in other species (cattle, pig, or horse). The following datasets were used: pregnant bovine endometrium day 15 [33], pregnant pig endometrium day 12 [34], and pregnant horse endometrium day 16 [35]. GSEA was carried out by the use of the GSEA web start tool [36]. The core-enriched genes (overlapping between canine DEGs and genes expressed in other species) were further analyzed by DAVID (6.8) to extract GO information.

Next, a multiple comparison of all datasets was performed and visualized as Venn diagrams using Venny 2.1 software [37] to identify an intersection between commonly up-regulated or down-regulated genes in the early pregnant uterus of cow, pig or horse, using the canine gene set as a background. The canine background gene set was based on the top 2000 genes with positive scores and the top 700 with negative scores of the ranked list used for GSEA analysis. To enlarge the number of overlapping genes, the dataset for the bovine species at day 15 of pregnancy was combined with that detected at day 16 of bovine pregnancy [33], and the horse gene set from day 16 of pregnancy was combined with a gene list of uterine genes detected at day 12 of equine pregnancy. The gene set of pig was not modified and was the same as for GSEA (i.e., pig endometrium day 12 [34]). Additionally, a gene list comprising a dataset generated using human endometrium during the window of implantation (WOI) [38] was included. Genes expressed in the dog and at least one another species were considered. The proportional overlap between genes expressed in dog and other species was calculated. The total numbers of genes included in this analysis were as follows: dog 2700 (2000up/700down), human (WOI) 2300 genes (982up/1318down), bovine (days 15 and 16 of pregnancy) 864 genes (586up/278down), pig (day 12 of pregnancy) 1659 genes (898up/761down), and horse (days 12 and 16 of pregnancy) 1928 genes (1013up/915down).

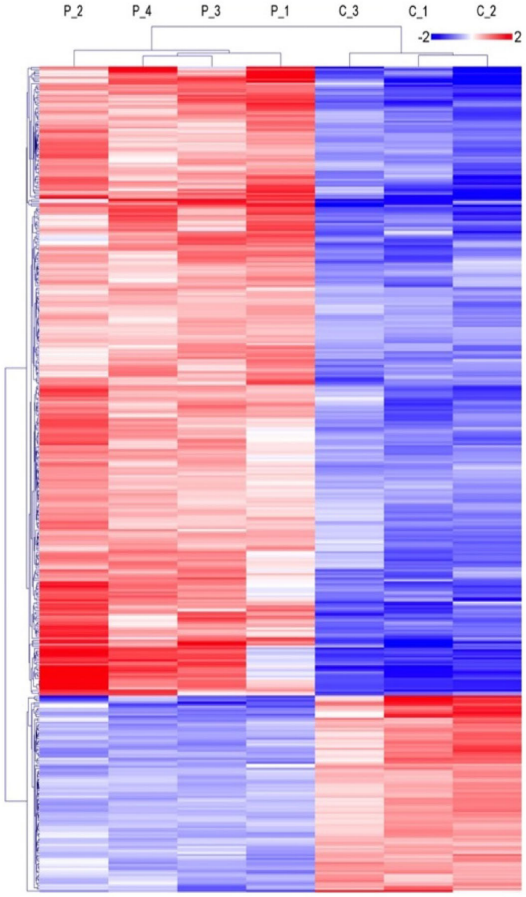
To identify gene clusters exclusively regulated in the dog, those canine genes that did not match in the aforementioned cumulative comparison with any of the other species (bovine, horse, pig, human) were used for DAVID analysis.

RNA isolation, reverse transcription, semi-quantitative (TaqMan) PCR and evaluation of data

Total RNA samples isolated from all uterine samples (n = 8 for early pregnant uteri and n = 6 non-pregnant controls) were used in semi-quantitative TaqMan RT-PCR in order to validate the expression of selected candidate genes detected by microarray analysis. RQ1 RNAse-free DNAse treatment (Promega) was applied following RNA isolation using TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer´s instructions. Complementary DNA (cDNA) corresponding to 200 ng RNA per sample and run was used for detection of each target gene. The reverse transcription (RT) reaction was performed with reagents from Applied Biosystems, Foster City, CA, USA. Detailed protocols were previously published [14, 39, 40]. TaqMan PCR reactions were run in duplicates with the Fast Start Universal Probe Master (ROX) (Roche Diagnostics AG, Rotkreutz, Switzerland). The negative controls consisted of autoclaved water instead of cDNA and the so-called RT-minus control [14, 40]. All analyses were performed in an automated fluorometer (ABI PRISM 7500 Sequence Detection System, Applied Biosystems). Customized canine-specific primers and 6-FAM- and TAMRA-labeled (TaqMan) probes were purchased from Microsynth, Balgach, Switzerland, and are listed in Table 1. The

Table 1. List of primers used for real-time (TaqMan) PCR.

Primer	Accession number	Primer Sequence		Product length (bp)
allograft inflammatory factor 1 (AIF1)	XM_532072.5	Forward	5´-CGA ATG CTG GAG AAA CTT GGT-3´	107
		Reverse	5´-TGA GAA AGT CAG AGT AGC TGA AGG T-3´	
		TaqMan Probe	5´-TCC CCA AGA CCC ATC TGG AGC TCA A-3´	
chemokine ligand 12 (CXCL12)	NM_001128097.1	Forward	5´-AGC CAA CGT CAA GCA TCT CA-3´	90
		Reverse	5´-CAC ACC TGT CTG CTG TTG TTC TTC-3´	
		TaqMan Probe	5´-AAC TGT GCC CTT CAG ATC GTG GCA A-3´	
chemokine ligand 16 (CXCL16)	XM_014113226.1	Forward	5´-CAG CGT CAC TGG CAG TTG TTA C-3´	105
		Reverse	5´-CGC TGA TAG ACT CGC AGA TGT T-3´	
		TaqMan Probe	5´-CCG CGG AGC TCA TGG CTC ATC-3´	
chemokine receptor 6 (CXCR6)	XM_846798.3	Forward	5´-GCT GAA GAG CCT GAC AGA TGT G-3´	91
		Reverse	5´-GCT GGC ATA GGC CCA AAA G-3´	
		TaqMan Probe	5´-CTG ATG AAC CTG CCC CTA GCT GAC CTG-3´	
chemokine receptor 7 (CXCR7)	XM_005635904.2	Forward	5´-CGG CAT GAT CGC CAA CTC-3´	94
		Reverse	5´-GAT GGC CAG GTT GAG GAT GT-3´	
		TaqMan Probe	5´-CCA AGA CCA CCG GCT ACG ACA CG-3´	
laminin alpha 2 (LAMA2)	XM_014113700.1	Forward	5´-AAA CCG GCT CAC GAT TGA G-3´	99
		Reverse	5´-AGT TGA ACG GTG GCG AAG T-3´	
		TaqMan Probe	5´-CCT GCT CTT CTA CAT GGC TCG GAT CAA-3´	
liver X receptor (LXR)	XM_014120969.1	Forward	5´-GGC CCT GCA TGC CTA CGT-3´	67
		Reverse	5´-CAT TAG CAT CCG TGG GAA CAT C-3´	
		TaqMan Probe	5´-TCC ACC ACC CCC ACG ACC GA-3´	
papilin proteoglycan-like sulfated glycoprotein (PAPLN)	XM_005623922.1	Forward	5´-GCT GAT GGG CAT CGT GTT C-3´	103
		Reverse	5´-TTC CAC GGT AGG CAC TAC ATG T-3´	
		TaqMan Probe	5´-CCA TAA CCT GCG GGC CGG AGA C-3´	
pappalysin2 (PAPPA2)	XM_537179.5	Forward	5´-ACG GGA TTG GTG CAG TGT GT-3´	90
		Reverse	5´-CCA GAG TGT CAG CAG TGA TGT TC-3´	
		TaqMan Probe	5´-ATC GTG TGT AAT TCC CCC TAG CGATCC TG-3´	
phospholipase A2 (PLA2G4A)	XM_005622454.2	Forward	5´-AGA GAA AGG GCC AGA GGA GAT T-3´	139
		Reverse	5´-GGT GAC AGG TTG TCC AGA GCT T-3´	
		TaqMan Probe	5´-CTA CAA CCC CCT TTT GCT TCT CAC ACC A-3´	
prostaglandin D2 receptor (PTGDR)	XM_848401.3	Forward	5´-CGC CTT CTG CCT GGT TTT-3´	101
		Reverse	5´-CCT CGT GCA TCA TCT GGA T-3´	
		TaqMan Probe	5´-CGC TGC CCT TCG CGG GCT-3´	
tissue inhibitor of matrix metalloproteinase-2 (TIMP2)	AF188489.1	Forward	5´ -CCT GGA CAT CGG AGG AAA GA-3´	99
		Reverse	5´ -TCC CAG GGC ACG ATG AAG T-3´	
		TaqMan Probe	5´ -CGG CAA GAT GCA CAT CAC CCT TTG T-3´	
indolamin 2,3-dioxygenase 1 (IDO1)	XM_532793.5	Forward	5´-TGA TGG CCT TAG TGG ACA CAA G-3´	116
		Reverse	5´-TCT GTG GCA AGA CCT TTC GA-3´	
		TaqMan Probe	5´-CAG CGC CTT GCA CGT CTG GC-3´	
GAPDH	AB028142	Forward:	5‘-GCT GCC AAA TAT GAC GAC ATC A-3´	75
		Reverse:	5‘-GTA GCC CAG GAT GCC TTT GAG-3´	
		TaqMan Probe	5‘-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3´	
18S rRNA	FJ797658	Forward	5´ -GTC GCT CGC TCC TCT CCT ACT-3´	125
		Reverse	5´ -GGC TGA CCG GGT TGG TTT-3´	
		TaqMan Probe	5´ -ACA TGC CGA CGG GCG CTG AC-3´	



Main functional terms:

Upregulated in response to free-floating embryos (314 genes):

- Extracellular region; enrichment score (ES): 10.77 (185 genes)
- Inflammatory response; ES: 7.34 (145 genes)
- Regulation of cell motion and cell migration; ES: 6.15 (93 genes)
- Angiogenesis; ES: 4.61 (71 genes)

Downregulated in response to free-floating embryos (98 genes):

- Focal adhesion; ES: 2.65 (10 genes)
- Cell morphogenesis and cellular component morphogenesis; ES: 2.13 (27 genes)
- Cell adhesion; ES: 1.93 (21 genes)
- Fibronectin type3; ES: 1.73 (7 genes)

Figure 1. Heatmap showing the microarray analysis of DEG in early pregnant (P1-4) canine uterus at days 10-12, and corresponding non-pregnant controls (C1-3). In total, 412 genes (FDR: 10%) were differentially expressed between the two groups. A total of 314 genes were up-regulated and 98 down-regulated. The main functional terms determined by DAVID analysis are presented for the identification of those functional terms affected by the presence of free-floating embryos in the canine pre-implantation uterus. The full list of DEG (FDR: 10%) is provided in Supplemental File 2.

efficiency of self-designed expression assays was validated as previously described [40] ensuring approximately 100%. Three reference genes were used for normalization of data (*GAPDH*, Cyclophilin A (*PPIA*), and *18SrRNA*). The canine-specific TaqMan Gene Expression Assay for PPIA was ordered from Applied Biosystems (Prod. No. Cf03986523-gH). For relative quantification, the comparative CT method (ΔΔCT method) was applied as previously described [28, 40]. An unpaired, two-tailed Student *t*-test was performed with GraphPad 3.06 (GraphPad Software, San Diego, CA, USA). A P-value < 0.05 was considered as statistically significant.

RESULTS

Analysis of microarray data

The canine uterine response toward the presence of free-floating embryos (days 10-12 of pregnancy) was characterized using a customized Agilent microarray assay. DEG between the two experimental groups (pregnant vs. non-pregnant group) were evaluated.

The number of detected probes passing the "well above background flags" filter was 44 253. These probes were summed up on the gene annotation level, resulting in a total of 15′668 genes (provided as Supplemental File 1). These genes, ranked according to their expression level, were later used for GSEA analysis. Applying FDR of 10% (i.e., adjusted P-value < 0.1), 412 DEG between the two groups were found (Supplemental File 2). A total of 314 genes showed higher and 98 showed lower expression in the early pregnant group compared to non-pregnant uteri. The expression patterns of DEG in the two experimental groups, together with associated main functional terms, are visualized using heatmaps (Figure 1). In one of the dogs allotted to the non-pregnant group, the expression pattern of DEG exactly matched that of early pregnant dogs, suggesting that one of the samples was not properly allotted; most probably, the embryo/embryos were missed during the flushing process. This sample was excluded from further analysis. The three highest fold changes (FC) in the pairwise comparison "pregnant vs. control" were for the following genes: *IPO9/IMP9* (7.7-fold), *ITIH4/GP120/PK120* (7.6-fold), and *NOV/CCN3/IGFBP9* (7.5-fold), while the three lowest expressed genes, i.e., suppressed in early pregnant uterus, were: marker of proliferation *Ki-67 (MKI67)* (-6.4-fold), *PAPPA2* (-5.2-fold), and *DIAPH3* (-4.5-fold).

Functional annotations and networks analysis

Functional terms enriched for DEG from both experimental groups were identified by DAVID analysis. The main functional terms (Figure 1) showed over-representation of genes in the gene set as represented by the enrichment score (ES); total numbers of genes in each cluster are indicated. An increased uterine feedback in response to free-floating embryos was noted for functional networks associated with extracellular region, inflammatory response, cell motion,

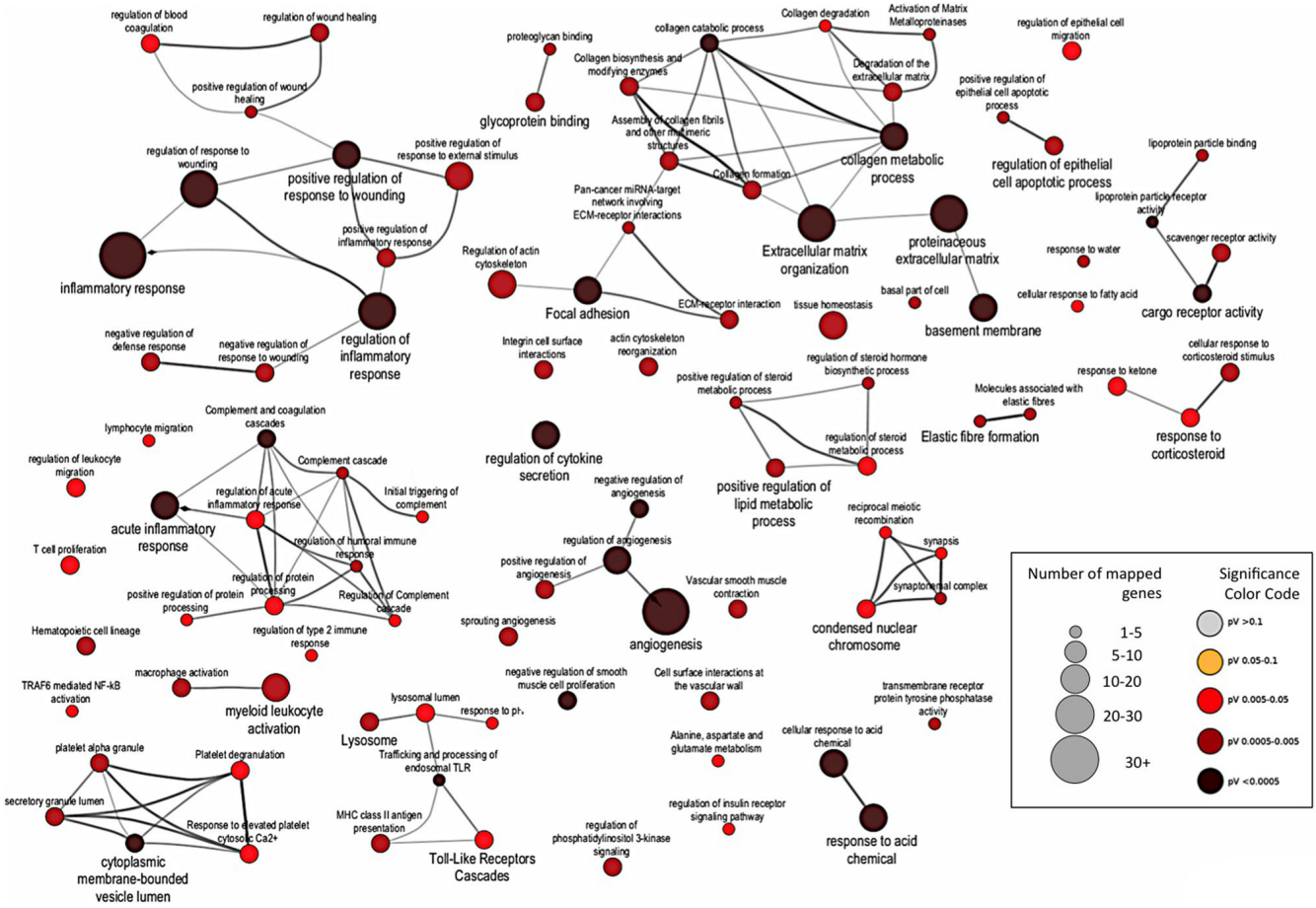


Figure 2. Cytoscape analysis of functional networks over-represented in canine uterus exposed to pre-implantation free-floating embryos. As input, DEG (FDR: 10%) were used (see Supplemental File 2). The redundant and noninformative terms were removed, and the resulting network was manually rearranged. For each network, the size of the node implies the number of genes, while the color intensity denotes the level of enrichment (see legend to illustration). Functional networks more highly represented in early pregnant canine uterus refer predominantly to ECM, immune response, and angiogenesis.

and migration and angiogenesis. The cluster "extracellular region" (ES 10.77) consisted of the following categories: glycoprotein, signal peptide, extracellular space, extracellular vesicle and secreted. Representative genes in this cluster were as follows: *ADAMTS1*, *BMP6*, *COL16A1*, *COL18A1*, *COL5A2*, *COL6A2*, *CXCL16*, *ECM1*, *IGF2*, *LAMA2*, *MMP2*, *MMP23B*, and *NOV*. The cluster "inflammatory response" (ES 7.34) comprised the following categories: defense response, response to stress, immune response, innate immune response, and positive regulation of immune system process. Representative genes were *AIF1*, *CSF1*, *CSF1R*, *ECM1*, *IDO1*, *IL15*, *IL16*, *IL1R1*, *LYZ*, *MMP2*, *NOV*, *PLA2G4A*, and *SERPINF1*. The cluster "regulation of cell motion and migration" (ES 6.15) consisted of the following categories: regulation of cellular component movement, regulation of locomotion, cell motility. Representative genes were as follows: *AIF1*, *BMP6*, *CCL15*, *CCL18*, *CSF1*, *CSF1R*, *CTSK*, *CXCL16*, *IL16*, *IL1R1*, *IL33*, and *SERPINF1*. The cluster "angiogenesis" (ES 4.61) comprised the following categories: regulation of vasculature development, regulation of angiogenesis, vasculature development, and blood vessel morphogenesis. Representative genes included *ADAMTS1*, *BMP6*, *COL18A1*, *COL5A2*, *ECM1*, *LAMA2*, *MMP2*, and *NFKB2*. The 98 down-regulated genes showed higher variability with lower ES (Figure 1). They could be connected to functional terms associated with focal adhesion, cell morphogenesis and cellular component morphogenesis, cell adhesion and fibronectin type 3. The term "focal adhesion" (ES 2.65) included categories related to regulation of actin cytoskeleton and platelet activation. Representative genes were *COL6A3*, *CRK*, *DIAPH3*, *FN1*, *ITGB1*, *MYLK*, *PARVA*, *PPP1CB*, *RRAS2*, and *TNC*. The cluster "cell morphogenesis and cellular component morphogenesis" (ES 2.13) consisted of categories: cell morphogenesis, cellular component morphogenesis, regulation of hydrolase activity, enzyme regulator activity, regulation of GTPase activity and regulation of catalytic activity. Representative genes were *ACVR1*, *CAB39*, *CDH2*, *CRK*, *FN1*, *HSPH1*, *ITGB1*, *NCAM1*, *PARVA*, and *ROBO1*. The cluster "cell adhesion" (ES 1.93) included the following categories: focal adhesion, cell-substrate adherens junction, anchoring junction, cell-cell adherens junction, actin binding, cadherin binding. Representative genes were, e.g., *ACVR1*, *CDH2*, *DIAPH3*, *FN1*, *HSPH1*, *ITGB1*, *LAYN*, *LIMS1*, *LRP8*, and *MYLK*. The cluster "fibronectin type 3" (ES 1.73) included genes such as *NCAM1*, *ROBO1*, *TNC*, *COL6A3*, *PAPPA2*, *MYLK*, and *FN1*.

For visualization of interactions between functional networks and their integration with DEG, Cytoscape analysis was performed (Figure 2). The main over-represented networks referred to extracellular matrix (ECM) (centered around matrix organization, focal adhesion, and basement membrane), immune response (centered around positive regulation of response to wounding, regulation of inflammatory response, and inflammatory response), and angiogenesis.

Using IPA on 412 DEG, the three highest over-represented canonical pathways were associated with acute phase response signaling ($P = 3.12E-07$; representative genes were: *C1R*, *HPX*, *IKBKB*, *IL33*, *IL1R1*, *NR3C1*, *C1S*, *RRAS2*, *FN1*, *NFKB2*, *C4A/C4B*, *ITIH4*, *TF*, *KLKB1*, and *SERPINF1*), liver X receptor *LXR* activation ($P = 1.44E-05$; representative genes: *ARG2*, *HPX*, *IL33*, *IL1R1*, *NR1H3*, *NFKB2*, *C4A/C4B*, *LYZ*, *ITIH4*, *TF*, and *SERPINF1*), and complement system ($P = 4.46E-05$, representative genes: *C1R*, *C1S*, *CD59*, *C4A/C4B*, *CFH*, and *ITGAM*) (Figure 3A).

The main functional terms derived from IPA analysis showed similar results, compared to DAVID analysis, with highly involved functional terms for cellular movement and immune cell trafficking (Figure 3B).

Expression of selected target genes by semi-quantitative RT-PCR

The expression of 13 selected target genes predicted to be differentially expressed was assessed by semi-quantitative (TaqMan) PCR. These were chosen related to the strongest over-represented functional networks present in the early pregnant canine uterus (i.e., ECM and immune modulation networks). Thus, increased expression of the following immunomodulatory genes was found in the early pregnant canine uterus in response to free-floating embryos: *AIF1* ($P = 0.002$), *CXCR6* ($P = 0.02$), *PTGDR* ($P = 0.001$), *IDO1* ($P = 0.02$), *CXCL16* ($P = 0.009$) and its respective receptor *CXCR7* ($P = 0.02$) (Figure 6). Pappalysin 2 *(PAPPA2)* was significantly decreased ($P = 0.04$) in the presence of free-floating embryos (Figure 6). The expression of CXCL12 and PLA2G4A, which was predicted to be elevated in early pregnancy, did not differ between the two groups in qPCR ($P > 0.05$; not shown). The validation of ECM-related genes displayed an increase for *LAMA2* ($P = 0.01$) and *TIMP2* ($P = 0.01$) (Figure 6), while *PAPLN*, which was expected to be expressed more highly did not differ significantly ($P > 0.05$) in qPCR (not shown). Following the canonical pathway analysis performed with IPA, the expression of *LXR* was evaluated and revealed an upregulation ($P = 0.002$) toward early pregnancy.

Comparison of canine differentially expressed genes (DEG) with genes expressed during early gestation in other species

Gene set enrichment analysis (GSEA)

The entire list of canine DEG derived from microarray analysis of uterine samples was compared with genes expressed in other species, i.e., bovine, pig, and horse (days 15, 12, and 16 of pregnancy, respectively) [33–35]. Therefore, genes listed in Supplemental File 1 (no FDR or *P*-value cut-off) were ranked according to a score based on log2 fold-change and *P*-value (see Methods) and compared to sets of genes either up-regulated (Figure 4A-C) or down-regulated (Figure 4D-F) in bovine, porcine, or equine uteri. For each gene set comparison, the enrichment profile, as well as the position of the matching gene on the ranked canine gene set, are indicated (Figure 4). Complete lists of core-enriched overlapping genes, identified by their official gene symbols, are provided in Supplemental File 3. To identify functional relations within the detected datasets between the dog and other species, the overlapping core enriched genes were used for DAVID analysis. The functional clusters determined by DAVID analysis are presented in Table 2. For easier interpretation of the data, information about the categories is given for each functional annotation (cluster). The over-representation of genes in the gene-set divided into functional clusters and categories, all represented by ES, including the total number of genes from the dataset is presented in Table 2. From all comparisons, only the contrast of down-regulated bovine genes at day 15 of pregnancy vs. all genes of the dog did not reveal over-represented functional categories, possibly relating to the small number of core enriched genes (n = 12 genes) (Figure 4D; Table 2). For this contrast, genes up-regulated in bovine endometrium at day 15 of pregnancy overlapped with genes that were also up-regulated in the dog (Figure 4A).

Most of the genes up-regulated in the porcine endometrium matched with canine down-regulated genes (Figure 4B; Table 2). In contrast, genes down-regulated in pig overlapped mostly with those genes up-regulated in the dog (Figure 4E; Table 2). As for the horse, whereas the up-regulated genes matched with up-regulated canine genes (Figure 4C, Table 2), down-regulated equine genes overlapped predominantly with genes up-regulated in the dog (Figure 4F; Table 2).

Venn diagrams

In addition, Venn diagrams were established to identify commonly expressed genes between early pregnant canine uterus and other species, including human during the WOI. This cumulative comparison of all datasets was performed with the background of the top 2000 positive and top 700 negative canine scores (listed in Supplemental File 4A). All datasets were analyzed for commonly (up- or down-) regulated genes (Figure 5A and B). Additionally all genes (up- and down-regulated) were cumulatively compared with each other (Figure 5C). In addition to the total number of overlapping genes, the proportional ratio of matching genes between the gene set from the different mammals (i.e., bovine, pig, horse and human) and the canine gene set is given. The full lists of overlapping genes identified in this analysis are presented in Supplemental File 5.

When all the up-regulated genes were compared with the top 2000 canine positively scoring genes (Figure 5A; Supplemental File 5), the highest proportional overlapping was found with the human and horse gene set (12.6%, 124 from a total of 982 genes, and 12.5%, 127 genes out of 1013, respectively). The cow matched with 11.6% (68 genes from a total of 586), and the up-regulated genes of pig matched with 8.9% (80 out of 898 genes) in the dog. As for the commonly down-regulated genes (Figure 5B, Supplemental File 5), the highest proportional overlapping with the top 700 down-regulated canine genes was again found for the human endometrium during the WOI. Here, 7.4% of all genes were overlapping (97 from a total of 1318 genes). The horse matched with the canine genes by 3.8% (35 out of 915), the cow overlapped with 2.9% (8 genes from a total of 278), and only 2% of genes (15 out of 716) overlapped for the pig. The combined analysis (lists of up- and down-regulated genes merged for each species and used for a cumulative comparison) showed the highest proportional overlap of identical genes with 15.7% for the porcine gene set (261 from a total of 1659 genes), 14.9% for the horse, (288 genes out of 1928), 14% for human (322 genes from a total of 2300) and 13.1% for cow (113 out of 864) (Figure 5C; Supplemental File 5). Here, the overall number of total gene hits was the highest in all three cumulative comparisons.

While 774 genes from the list of 2700 canine genes up- and down-regulated matched with genes expressed in other species in the cumulative comparison, 1926 canine genes were without a match (Supplemental File 4B). These unpaired genes specifically expressed in the dog were used for functional annotation clustering by DAVID to reveal their molecular functions. The identities of functional annotation clusters and categories, along with the respective ES and total number of genes in clusters, are presented in Table 3. The main

Figure 3. IPA of canine DEG (early pregnancy vs. non-pregnant). (A) The canonical pathways analysis with the output gene sets ranked according to log (*P*-value). The overlapping of gene sets and the trend of the status (up- or down-regulated) are indicated. (B) The main functional terms identified by IPA were determined based on the identified canonical pathways.

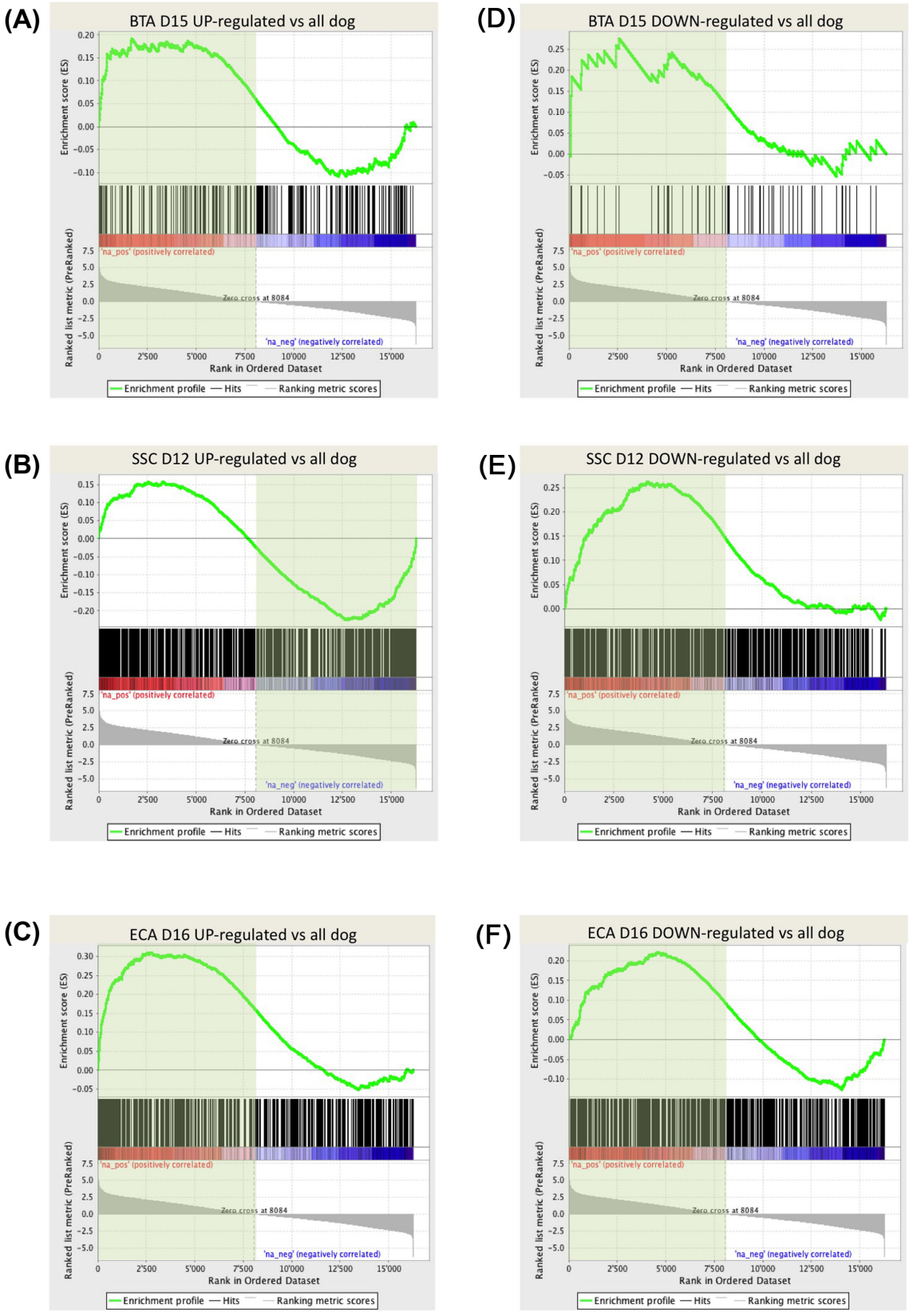
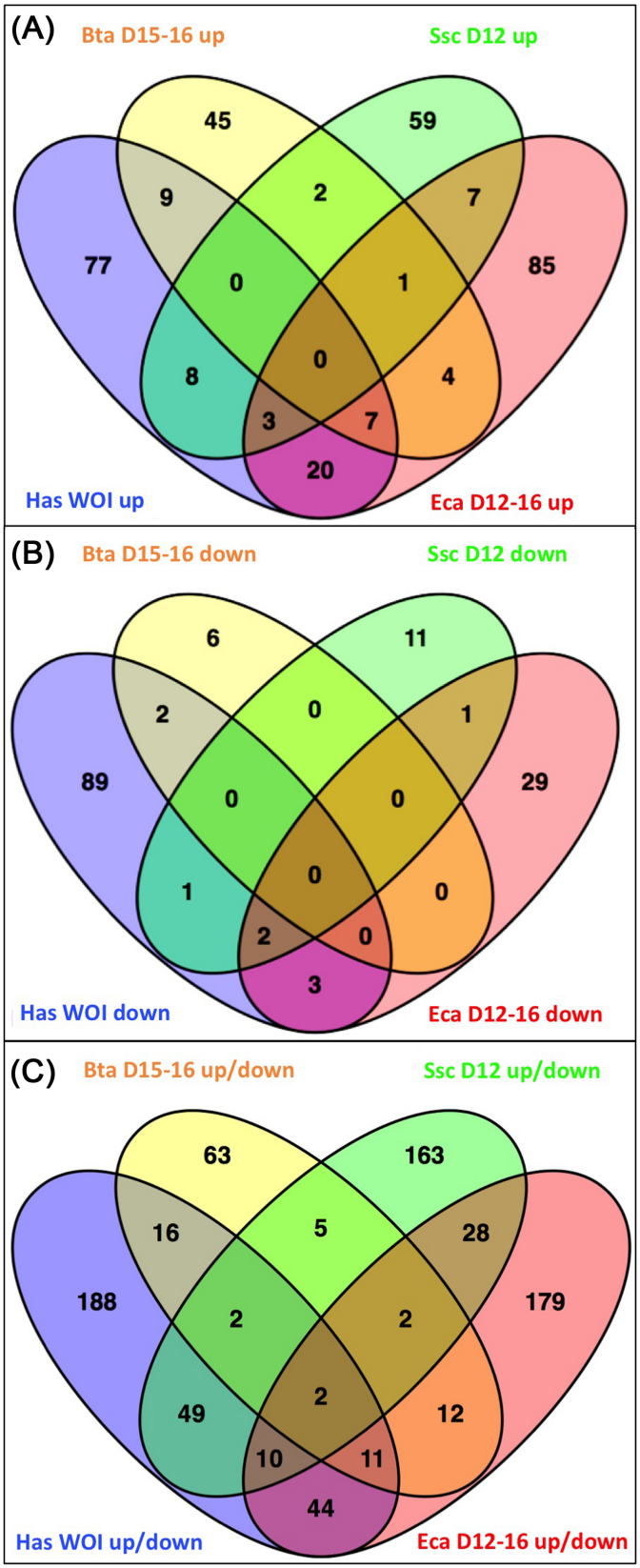


Figure 4. GSEA. The entire set of genes detected in canine uterus was ranked according to their expression levels (a score calculated from the log2 fold change and the *P*-value; see Methods). The ranked list was compared to sets of up-regulated (UP-regulated) genes (A-C) or down-regulated (DOWN-regulated) genes (D-F) from bovine (Bos taurus/BTA) uterus at day 15 of pregnancy (A and D), swine (Sus scrofa/SSC) uterus at day 12 of pregnancy (B and E) and horse (Equus caballus/ECA) uterus at day 16 of pregnancy (C and F). Enrichment scores and quantitatively strongest overlapping with the canine gene set are indicated. For details see the text. The list of overlapping genes is provided as Supplemental File 3.

Table 2. Results of functional annotation clustering analysis performed by DAVID software.

Gene set compared with canine gene set (ranked according to expression level)	Cluster	Category	Enrichment score (ES)	number of genes in the cluster
bovine upregulated (day 15 of pregnancy) 30 core-enriched genes	ion transport	carboxylic acid, organic anion transport	1.42	3
	epidermal growth factor (EGF)	EGF-like calicium binding, EGF-like domain, hydrolase	1.36	10
	extracellular matrix (ECM) signal	signal, ECM vesicle and exosome, ECM region, glycoprotein	1.32	15
	lipid transport	lipid transport, lipid localization	1.30	3
pig upregulated (day 12 of pregnancy) 245 core-enriched genes	cell cycle, division and mitosis	cell cycle process, mitotic cycle process, nuclear fusion, chromosome segregation, chromosome, regulation of mitotic cell cycle	6.27	100
	intracellular	intracellular organelle, intracellular membrane-bound organelle, cytoplasm	5.54	189
	cell selforganisation	microtubule cytoskeleton, non-membrane bound organelle, cytoskeletal part	4.24	73
pig downregulated (day 12 of pregnancy) 191 core-enriched genes	metal ion	transition metal ion homeostasis, cellular transition metal ion homeostasis, cellular iron ion homeostasis	2.04	7
	ECM	ECM-region, extracellular vesicle/exosome	1.97	70
	maturation	developmental and cell maturation	1.90	9
	mitogen-activated protein kinase kinase kinase (MAP3K)	MAP3K activity, activation of protein kinase activity	1.69	6
horse upregulated (day 16 of pregnancy) 142 core-enriched genes	ECM	ECM-region, ECM exosome, membrane-bound vesicle	6.50	95
	ECM-space	ECM-space	4.30	21
	blood vessels	blood vessel morphogenesis/development, regulation of angiogenesis, circulatory and vasculatory system development, regulation of anatomical structure morphogenesis	2.70	31
	immune system	positive regulation of immune system process, complement activation, response to external stimulus, regulation of protein activation cascade, regulation of defense response, regulation of B cell mediated immunity, regulation of humoral immune response	2.48	52
	lipoprotein	cargo receptor activity, lipoprotein particle receptor activation	2.25	4
	endocytosis	phagocytosis, endocytosis, vesicle mediated transport	2.17	26
	organ development	single-multicellular organism process, regulation of multicellular organismal process, system development, anatomical structure morphogenesis	2.01	54
horse downregulated (day 16 of pregnancy) 188 core-enriched genes	Golgi apparatus	Golgi apparatus, endomembrane system	2.31	33
	metabolic process	cellular amine metabolic process, catabolic process, small molecule catabolic process	2.09	8

Core-enriched genes derived from GSEA were used as input. For this, the entire canine gene set (no FDR, no *P*-value correction), ranked according to gene expression levels, was used as background and compared with genes differentially expressed in other species (either up- or down-regulated). The clusters of main selected over-represented functional terms are shown. The categories comprising each cluster have been additionally named. The over-representation of genes in gene sets is represented by respective ES. The number of genes in each cluster is given.



Proportional overlap [%] between genes expressed in dog and other species:
Bta = 11.6%
Ssc = 8.9%
Eca = 12.5%
Hsa = 12.6%

Proportional overlap [%] between genes expressed in dog and other species:
Bta = 2.9%
Ssc = 2.0%
Eca = 3.8%
Hsa = 7.4%

Proportional overlap [%] between genes expressed in dog and other species:
Bta = 13.1%
Ssc = 15.7%
Eca = 14.9%
Hsa = 14.0%

Figure 5. Venn diagrams showing the intersection between genes differentially expressed in the presence of pre-implantation embryos in different domestic animal species are presented using the canine gene set as reference. (A) Overlapping of the top 2000 up-regulated (2000 best positive scores) canine genes with up-regulated genes in other species; (B) comparison of the top 700 down-regulated (700 best negative scores) canine genes with genes down-regulated in other species; (C) cumulative analysis of the canine genes with genes up- and down-regulated in other species. Hsa WOI = Homo sapiens during the window of implantation (WOI), Bta = Bos taurus (days 15–16 of pregnancy), Ssc = Sus scrofa (day 12), and Eca = Equus caballus (days 12 and 16). The list of overlapping genes is provided as Supplemental File 4.

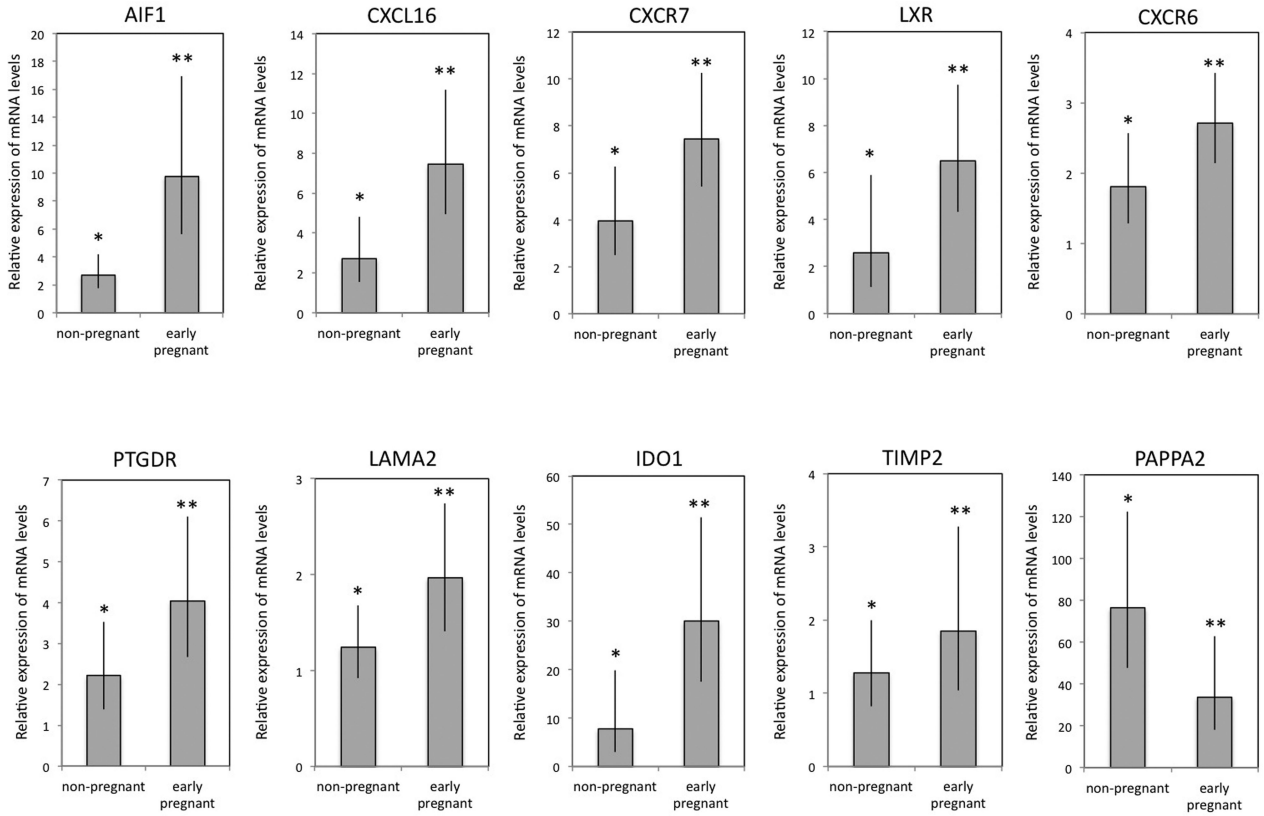


Figure 6. Expression of selected target genes as determined by real-time (TaqMan) RT-PCR. *AIF1* = allograft inflammatory factor 1, *CXCL16* = chemokine ligand 16, *CXCR7* = chemokine receptor 7, *LXR* = liver X receptor, *CXCR6* = chemokine receptor 6, *PTGDR* = prostaglandin D2 receptor, *LAMA2* = laminin alpha 2, *IDO1* = idolamin 2,3-dioxygenase 1, *TIMP2* = tissue inhibitor of matrix metalloproteinase-2, *PAPPA2* = pappalysin2. An unpaired, two-tailed Student *t*-test was applied. Bars with different asterisks differ at $P = 0.001$ (*PTGDR*), $P = 0.002$ (*AIF1, LXR*), $P = 0.009$ (*CXCL16*), $P = 0.01$ (*LAMA2, TIMP2*), $P = 0.02$ (*CXCR7, CXCR6, IDO1*), $P = 0.04$ (*PAPPA2*). Numerical data are presented as geometric means Xg ± geometric standard deviation (SD).

Table 3. DAVID analysis from 1926 genes that did not overlap in the contrast: canine genes (2000 top positive/700 top negative) vs. gene lists from other species (corresponding to Figure 5).

Comparison	Cluster	Category	Enrichment score (ES)	number of genes in the cluster
1926 genes that did not pair in the contrast 2700 canine genes (up- and downregulated) versus other species (up- and downregulated)	mitochondrion	mitochondrion, mitochondrial matrix	6.79	159
	DNA repair	DNA repair, DNA damage	2.75	55
	helicase	helicase, helicase C-terminal, ATP dependent RNA helicase activity, oligosaccharide-binding, helicase-associated domain	2.36	27
	zinc-finger	zinc, zinc-finger, metal binding	1.93	353

The clusters of main selected over-represented functional terms are shown. Additionally, functional categories comprising the main clusters are named. The over-representations of genes in the gene set are represented by the ES, the number of genes in each cluster is given.

functional clusters related to mitochondrion (ES: 6.79; 159 genes), DNA-repair (ES: 2.75; 55 genes), helicase (ES: 2.36; 27 genes) and zinc-finger (ES: 1.93; 353 genes).

Discussion

Because there is no luteolysis in the absence of pregnancy, the dog clearly lacks an endocrine signal triggering a biochemical anti-luteolytic cascade, which is present in other species to modulate uterine function during the critical early time point of pregnancy establishment. This is reflected in species-specific responses of the uterus to pre-implantation embryos. Accordingly, in our previous study we showed that in the dog, biochemical changes precede morphological modifications in the early pregnant uterus [24, 25]. The possible underlying biological mechanisms have been further explored herein using microarray analysis of uterine transcriptomes. Uteri collected between days 10-12 of pregnancy were used. It is

noteworthy that, in the dog, development of embryos and their oviductal passage are slow. It takes as long as 7-10 days for embryos to reach the tip of the uterine horns [41, 42] and are usually then at the morula or early blastocyst stages [41]. Invasion of the uterine epithelium occurs normally around days 17-18 after fertilization [43, 44]. In our study, assuming that seminal bioactive factors would have an effect on the initiation of pregnancy by modulating the uterine endocrine milieu, dogs inseminated but determined to be non-pregnant were used as controls.

Because of the prolonged oviductal transit of embryos, any disturbance in their early development leading to pregnancy failure would not originate in the uterus. This approach allowed us to determine embryo-driven effects within a similar uterine environment. The effects of seminal plasma on the canine uterus (i.e., comparing non-mated dioestric bitches with those exposed to seminal plasma) were not separately studied herein, but certainly merit attention in the future.

Microarray analysis of responses of the canine uterus to early pre-implantation embryos

The major biological processes activated by the presence of early pre-implantation embryos in the canine uterus are indicated by the main functional terms detected associated with over-represented genes. Thus, the majority of the 412 DEG was linked to ECM and cell signaling, secretory activity and matrix-cell interactions involved in cell motion and migration. The second important group was represented by inflammatory response. Higher variation and less involved genes were found among functional terms suppressed when free-floating embryos are present. These were, however, specifically linked to cell-to-cell contacts including focal and cell adhesion and fibronectins activity. These observations were confirmed by the Cytoscape analysis of functional networks and were further fulfilled by over-represented pathways detected by IPA. In particular, acute phase response signaling, the complement system and pathways associated with adhesion and diapedesis of immune cells were strongly represented.

Regarding ECM components, the initialization of structural reorganization of the uterus driven by the presence of embryos in the dog was characterized by higher representation of genes encoding for several collagen compounds (e.g., *COL16A1* or *COL18A1*). It seems also that modifications of the uterine ECM, prior to embryo attachment and trophoblast invasion, might be affected by functional changes in integrins signaling because the respective pathways were either down-regulated (e.g., *RRAS2*, *ITGB1*, *LIMS1*) or up-regulated (e.g., *ARPC1B*, *ITGAM*) in the presence of early embryos. Integrins are transmembrane receptors that interconnect cells and build links between cell and ECM proteins such as laminin or collagens [45]. Disruption of their functionality may lead to infertility [46]. A modulated, increased expression of some integrins, such as -alpha2b, -beta2 and -beta3, was previously shown in the canine early pregnant uterus [47].

Another important adhesion molecule induced by the presence of canine pre-implantation embryos was laminin alpha 2 (*LAMA2*). This glycoprotein is a constituent of the *lamina basalis* [48] and is also produced by human decidual cells [49, 50]. It could thus be seen as an early indicator of the ongoing decidualization process in the dog, even if the samples used for this study were collected before the morphological differentiation of stromal cells into decidual cells becomes visible [24, 25].

As indicated elsewhere, among the functional terms, the representation of which was suppressed by the presence of early embryos, were those related to fibronectins, in particular to FN3. Fibronectins are ECM glycoproteins interacting with membrane-bound proteins such as integrins, fibrins, or actins. Specifically, the FN3 domain is evolutionarily well conserved and can be found in a variety of extracellular proteins. Thus, NCBI's Conserved Domain Database [51] annotates the biomolecular sequence of the conserved protein domain of FN3 (cd00063) in collagens and some cell adhesion molecules like receptor signaling family leucine-rich repeat transmembrane protein (FLRT) or ITGB4. Its presence has also been confirmed in the structural protein FN1, the expression of which is modulated by pregnancy-related hormones, e.g., P4 [52]. In the rat, the early pregnancy-related uterine decrease in fibronectin has been linked to the differentiation of stromal cells during the decidualization process [53]. Accordingly, the suppression of the respective FN domain found in our study in the early pregnant canine uterus could also be seen as a further sign of the ongoing decidualization process initiated by the presence of embryos.

While the previously mentioned molecules (i.e., integrins and FN) are considered stable components of the ECM, IPA also indicated an involvement of enzymes that modulate the composition of the ECM, like inhibitors of matrix metalloproteinases (TIMPs), in the early embryo-maternal communication in the dog. Considering the high representation of the immune system in our dataset, it is worth mentioning that some of these MMPs can be stimulated by proinflammatory cytokines such as IL-1 beta, IL-6, and IL-8 [54, 55]. So far, the presence and activity of MMP2 and MMP9 have been confirmed in the early pregnant uterine compartments of the dog [56, 57].

The expression of MMPs-modulating TIMPs and their involvement in embryo-maternal communication in the dog remain to be elucidated. Here, based on the results obtained from our pathway analysis, the expression of *TIMP2* was examined. TIMP2 is among the most potent regulators of MMPs activity and was shown to be positively regulated by P4 [58, 59]. In our experiments, its uterine expression was enhanced by the presence of free-floating embryos in the uterus. Future studies should focus on the early initiation of TIMPs expression and function in the canine uterus, because these proteins could be part of a balancing system by weakening the effects of cytokine-enforced activation of MMPs. However, this hypothesis needs further verification. Interestingly, from the 98 genes down-regulated in the canine uterus in response to the presence of embryos, among the most highly suppressed genes (5.2-fold suppression) was *PAPPA2*, also referred to as pregnancy-associated plasma protein A2. PAPPA2 acts as a metalloproteinase specific for insulin-like growth factor-binding proteins, and is thereby known to modulate the bioavailability of IGFs [60]. Besides their mitogenic activity [61–63], IGFs are prominent markers of decidualization and are important regulators of embryonic development and fetal and placental growth [64, 65]. Both IGF1 and IGF2 and their main receptor IGFR1 are present in the pre-implantation canine uterus [24]. IGFs appear also to be involved in species-specific canine decidualization [39]. It thus appears plausible that by being modulated by the presence of embryos, PAPPA2 is also involved in coordinating the local availability of IGFs in the dog. However, its involvement in the decidualization process and preparation for trophoblast implantation and invasion needs to be investigated.

Cumulatively, our findings emphasize the importance of pre-attachment canine embryos in biochemical modulation of uterine ECM components in preparation for implantation and placentation in this species. It is likely that, during these processes, not only the maternal stroma cells but also changes in the composition of the

uterine ECM have an impact on canine-specific process of decidualization. This is further supported by the top score genes detected by our microarray analysis, i.e., genes responding with the highest individual fold-enrichment to the presence of free-floating embryos (*IPO9*, *ITIH4*, and *NOV*). Their functionality strongly relates to biological processes of cell differentiation, proliferation, and matrix organization as indicated by DAVID and IPA, as well as being involved in immune processes. Thus, IPO9 is a part of the nucleocytoplasmic transport system by regulating the localization and functions of substrate proteins as a generic nuclear localization sequences receptor and signal transducer [66, 67]. One of the reported signaling pathways, the Wnt/Wingless pathway, is possibly associated with decidualization [68]. By regulating the expression of some interferons, e.g., IFN epsilon [69, 70], IPO9 also exerts immunomodulatory effects. Some other importins, e.g., IPO13 and IPO5 alpha, play crucial roles in uterine cellular differentiation and proliferation [71–73]. In knockout mice lacking IPO5 alpha expression, uterine stimulation with exogenous gonadotropins results in hypertrophy, similar to the P4 receptor-deficient phenotype [71]. The interalpha trypsin inhibitors (ITIH) function as protease inhibitors and are thereby involved in maintenance of ECM [74]. More specifically, ITIH4 is an acute phase protein, and besides involvement in systemic inflammation [75], it seems to play roles during pregnancy, e.g., in pigs, where it has been implicated in the maintenance of uterine surface glycocalyx [76]. Some other functions described for ITIH4 include immunomodulatory properties due to binding and suppression of the phagocytic activity of granulocytes [77].

Finally, a member of the family of CCN genes (connective tissue growth factor, cysteine-rich protein, and nephroblastoma overexpressed), NOV plays roles in several biological processes regulating cell functions such as adhesion, migration, chemotaxis, cell proliferation, and differentiation [78, 79]. Some of its functions are mediated by direct binding and activation of integrin receptors [80, 81]. The importance of NOV during pregnancy, possibly due to its angiogenic and tissue remodeling properties, is underlined by the fact that its decreased expression has been implicated in the early onset of pre-eclampsia [82].

Apart from *PAPPA2*, the evaluation of the top down-regulated scores, i.e., the most negatively affected genes in our microarray, revealed genes linked to cellular proliferation and maintenance of cellular cytoskeleton (*Ki67/MKI67* and *DIAPH3*). Ki67 is a well-known nuclear protein frequently used as a marker of proliferation [83]. It is present during all active phases of the cell cycle except the G0-phase [84]. Interfering with its function results in inhibition of RNA synthesis [85].

Diaphanous related formin (DIAPH3) in turn has not yet been directly connected to pregnancy, but its involvement in some basic, yet pregnancy-relevant mechanisms, has been shown. Thus, it is involved in actin remodeling, erythropoiesis, vesicle trafficking, bleb formation, filopodium formation, cell invasion, and cytokinesis [86–98].

Although not targeted toward suppression of luteolysis, species-specific immunomodulation indeed seems to play a major role in the canine early embryo-maternal conversation. The involvement of acute phase factors in this cross-talk was also implied in earlier studies, in which elevated levels of some acute phase response molecules, e.g., fibrinogen and serum C-reactive protein-like CRP-LI, were found in the serum of pregnant bitches [99–101]. These were, however, associated with implantation and placentation and were observed concomitantly with increasing placental RLN levels. The breakdown and remodeling of uterine tissues during early placentation were also associated with increased expression and activation of acute phase response in other species such as rats, in which particularly the AGP was strongly increased [102]. However, similar to the situation found in dogs, since they act as mediators of inflammation, triggered, e.g., by some infections, these acute phase proteins cannot be used as highly specific markers of pregnancy.

Regarding the pre-implantation uterus, our data are in agreement with previous reports of increased availability of transcripts encoding for several cytokines possibly involved in maternal recognition of pregnancy in dogs, such as *IL2*, *IL4*, *IL10*, *IFN gamma*, *TGF beta*, and *LIF* [57]. Concomitantly, other immunomodulatory factors, such as *GM-CSF*, *IL1 beta*, *IL6*, *IL8*, and *CD4*, were present in free-floating embryos [57]. Additionally, in our recent studies, we found an increased abundance of *PTGS2* (*COX2*) and *PTGES* transcripts in free-floating-hatched blastocysts compared with unhatched ones [24]. These data also fit very well with the IPA results presented herein, pointing toward activation of the IL10-, IL4- and IL6-pathways in the early pregnant canine uterus. Some other relevant immunoreactive genes from the respective functional terms "inflammatory response" and "defense response" and "innate immune response," which were highly represented in our microarray data from the canine early pregnant uterus were *CSF1*, *CSF1R*, *AIF1*, *IDO1*, *IL15*, *IL16*, *IL1R1*, and *LYZ*.

Macrophage colony-stimulating factor (M-CSF/CSF1) is a cytokine that, through binding to its receptor CSF1R, induces differentiation, proliferation, and survival of macrophages. These cells, besides mediating immune responses, contribute to tissue remodeling during mammalian pregnancy [103, 104]. Their roles also include phagocytosis of apoptotic cells that occur during the remodeling processes and trophoblast invasion, thereby decreasing local inflammatory responses [105]. Lysozyme (LYZ) is an antibacterial enzyme catalyzing the hydrolysis of peptidoglycans. It is present in different exocrine secretions such as saliva, milk, and mucus [106]. Additionally, it is present in cytoplasmic granules of macrophages and polymorphonuclear neutrophils [107]. As part of the innate immune system, LYZ seems to provide protection against uterine infections [107]. Its uterine secretion and function is positively influenced by P4 [108].

Moreover, among the selected genes investigated in our study by semi-quantitative PCR and belonging to the immune response, we were able to confirm stimulatory effects of the presence of canine embryos on uterine expression of transcripts encoding for *IDO*, *AIF1*, *CXCL16* and its receptor *CXCR6* and *CXCR7*.

Indolamine 2,3-dioxygenase, a versatile and highly conserved protein in mammals, is expressed by a wide variety of immune cells, e.g., dendritic cells, monocytes, and macrophages [109]. Through catabolism of the essential amino acid tryptophan, it modulates the behavior of T-cells [110]. It thus has immunomodulatory and, in part, immunosuppressive functions, when the withdrawal of T-cell activity is considered [111]. An interesting aspect of IDO in both immunomodulation and immunosuppression is its central role in the activation of regulatory T cells (Treg). Namely, the IDO-derived metabolic products of tryptophan stimulate production of Treg [112]. Regarding the reproductive organs, IDO is known to be secreted by human decidual cells [113] and its immunomodulatory activity during pre-implantation and implantation stages of pregnancy have been implied, e.g., in humans [114]. Also, its involvement in regulating trophoblast invasion was suggested because inhibition of IDO function leads to embryo rejection by T lymphocytes [115]. Besides T-cells, IDO is capable of suppressing activated natural killer (NK) cells, which constitute the highest

proportion of immunomodulatory cells in human decidua during the first trimester of pregnancy [116–118]. Thus, at least in species displaying an invasive type of placentation, IDO is needed for implantation. In view of this, we felt prompted to assess its expression and, thereby, its potential involvement in early embryo-maternal communication in dogs. The quantity and types of immune cells in the canine placenta still need to be elucidated, but the elevated mRNA levels of IDO1 in the early pregnant group of dogs seem to be related to a local immunosuppression and/or modulation. The function of IDO in canine pregnancy therefore needs to be investigated further.

Similar to IDO, allograft inflammatory factor 1 (AIF1) is also an evolutionarily old and highly conserved protein in mammals. AIF1 is a cytosolic protein that plays many roles, e.g., in regulating immune responses, proliferative activities, and vasculogenesis [119–123]. It can be found in macrophages, including those residing in the uterus. [124, 125]. Here, we detected significantly elevated uterine levels of AIF1 in the early pregnant canine uterus, suggesting possible physiological functions of AIF1 during maternal responses to the presence of pre-attachment embryos in this species.

The strong, immunomodulatory uterine activity, driven by the presence of embryos, was further underlined by the increased expression of several chemokines. These are needed as regulators of maternal receptivity and embryo implantation [126, 127] and were also shown to stimulate cell proliferation and trophoblast invasion [128, 129]. CXCL16 is a cytokine belonging to the CXC chemokine family. Its receptor, CXCR6, can be found on immune cells such as T cells and natural killer cells [130]. Binding of CXCL16 to its receptor CXCR6 will lead to cell migration [130]. In the human placenta, cytotrophoblast cells coexpress CXCL16 and CXCR6, which is thought to induce their proliferation and invasion in an autocrine manner [131, 132]. Similarly, CXCR7 and its ligand CXCL12 were implicated in supporting the invasive properties of human cytotrophoblast [133]. It therefore seems plausible that this chemokine-receptor-axis is up-regulated in the pregnant canine uterus to induce proliferation, and to modulate the immune milieu in preparation for implantation and placentation.

Due to the crucial role PG plays in reproduction, including the canine reproductive system, the increased expression of PTGDR driven by the presence of embryos attracted our attention. Its ligand, PGD2, is produced in the uterus and is secreted by endometrial and myometrial compartments, including decidua, e.g., in humans [134, 135]. Acting in a receptor-dependent manner, during pregnancy PGD2 functions as a chemoattractant to recruit immune cells to the feto-maternal interface [136, 137], and inhibits antigen presentation of dendritic cells to T cells [138]. Taking this into account, and its strong expression driven by the presence of embryos, PTGDR and its ligand PGD2 appear to be attention-worthy targets for investigating the species-specific mechanisms of embryo-maternal recognition in the dog.

Interestingly, the activation of the nuclear receptor LXR pathway in early pregnant canine uterus was indicated by IPA analysis. LXR is a heterodimeric binding partner of another nuclear receptor, PPAR gamma [139], and is thus linked to metabolic pathways, such as glucose and cholesterol metabolism, insulin metabolism, and adipogenesis [140, 141]. On the other hand, PPAR gamma uses PG as endogenous ligands and can thus participate in PG-mediated biological processes, including immunomodulatory functions [142, 143]. The role of PPAR gamma has also been implied during canine pregnancy, and while it was localized solely to fetal trophoblast cells, its functions were predominantly linked to the processes of implantation, trophoblast invasion, and placentation [40].

Correlation of canine differentially expressed genes to different species

Being interested in differentiating between the common and species-specific biological pathways involved in embryo-maternal communication during establishment of pregnancy in the dog, pairwise comparisons were established with previously published datasets from other animals. The gene lists from other species were derived from the time points of pregnancy when the anti-luteolytic signals are initialized in these species. These single interspecies comparisons were performed using the whole canine gene set (both gene expression directions, up- and down-regulated), matching it with gene sets that were filtered according to their expression (either up- or down-regulated) in other species. These analyses were set up to identify if similar pathways are utilized in dogs as in other species. The lowest ES were obtained in comparison with the bovine uterus. When canine and porcine gene sets were compared, higher ES were found, however, most of the overlapping genes showed opposite patterns of expression, indicating different embryo/conceptus-mediated uterine responses prior to implantation. Thus, DAVID analysis of genes derived by GSEA related the porcine up-regulated genes to cell cycle, cell division and mitosis. This contrasted with down-regulated genes related to functional terms associated with enzyme activity, ECM, maturation, and MAP3K activity, which mapped with genes up-regulated in the dog. This reverse correlation can be plausibly explained by the species-specific estrogen-related signaling in the pig in which the embryo-controlled anti-luteolytic switch from endocrine to paracrine PGF2 alpha secretion is initiated between days 10-12 of pregnancy [6, 34, 144]. In the dog, no pregnancy-related estrogen increase occurs [19], and the estrogenic capability of canine embryos remains unknown. It is notable that, as mentioned elsewhere, in bitches P4 appears to be the only luteal steroid required for the establishment of pregnancy [145, 146]. Furthermore, the highest ES were noted when canine uterine genes were compared with genes up-regulated in the mare. Here, the commonly up-regulated genes belonged to functional terms related to secretory activity, ECM, and angiogenesis. Nevertheless, as for the pig and cow, the down-regulated equine genes matched those genes that were up-regulated in the dog with enriched functional terms relating, e.g., to Golgi apparatus and metabolic processes. This indicates that, although sharing some similar regulatory pathways regarding structural remodeling of the uterus, nevertheless, canine and equine uteri differ markedly at the level of secretory activity. Moreover, with regard to similar expression of ECM-related factors between the dog and horse, most probably, the considerably delayed implantation observed in the horse needs to be considered. In the horse, implantation does not occur until around day 37 [12]; therefore, the blastocyst is dependent on histiotrophic support.

Going one step further, in addition to the pairwise interspecies comparisons, accounting for the expected differences arising from species-specific anti-luteolytic strategies and different types of implantation and preparations for placentation, commonly regulated genes in different species were determined in multiple comparisons. Canine genes were used as references for preparation of Venn diagrams. In contrast to the single interspecies comparisons by GSEA, all gene sets (canine, bovine, porcine, equine, and human) were filtered according to their gene expression (up- or down-regulated) and compared with each other. This allowed us to identify commonly

up- or down-regulated genes. Additionally, all gene sets (regardless of gene expression direction of change) were used to identify potentially conversely regulated genes between species during the early embryo-maternal dialog. Again, a high correlation was found between genes commonly expressed (up- or down-regulated) in the canine and equine uterus.

For this comparison, the human-derived gene set was included, resulting in even higher correlation with the canine uterus. These human genes were determined during the WOI [38], which lasts for 6-10 days after ovulation and is defined as the period when the uterus is receptive to implantation of the free-floating blastocyst [147]. During this time, the estrogen- and progesterone-primed endometrium is undergoing distinct morphological and histological changes, and maternal decidual cells are formed, the latter even in the absence of an implanting blastocyst [148, 149]. Our results thus seem to be linked to the P4-mediated effects and ongoing decidualization in the dog in preparation for implantation, when morphological decidualization of stromal cells becomes obvious [25]. Preparation for the invasive type of placentation may also play an important role.

Noticeably, the more species that were overlapped, the fewer genes were found to be commonly expressed. Interestingly, however, higher numbers of genes overlapped when they were compared regardless of their expression patterns (up- or down-regulated). This implies that different strategies within similar regulatory pathways are utilized for facilitating implantation in different mammals.

In this regard, we were able to identify some genes represented in all compared species. These were *PENK* and *CYP26A1*. PENK was up-regulated in bovine, horse, and dog, but down-regulated in human and pig. Its exact involvement in pregnancy is not yet completely understood, but there are indications that PENK, as part of the opioid system, could play a role in maternal adaption to pregnancy and in supporting embryonic and fetal growth by modulating analgesia and regulating uterine motility [150, 151]. CYP26A1 is a microsomal enzyme responsible for regulating the cellular level of retinoic acid, which is involved in modulating gene expression in embryonic and adult tissues [152, 153]. In the gene sets applied here, CYP26A1 was up-regulated in human, cow, and dog, while it was down-regulated in horse and pig. It seems that functions of both genes in the uterine preparation for implantation are species-specific, and possibly also stage of pregnancy-specific, which is reflected in their divergent expression patterns among the different animals.

Interestingly, we found 1926 genes that were exclusively expressed in the canine pre-implantation uterus and, thus, did not overlap with genes expressed in other species. Most of them were specifically linked to mitochondrial functions, transcriptional activity of the genome and metabolic activities associated with DNA repair. Also, these energy- and protein-secretion-related functional terms could be related to other canine-specific up-regulated terms associated with the remodeling processes driven by the presence of embryos within the canine pre-implantation uterus.

Here, for the first time, deeper insights into species-specific pre-implantation embryo-maternal interaction in the dog are presented. Despite being devoid of a classical anti-luteolytic signal, profound biochemical changes were found, indicating the activity of biological mechanisms which are generally linked to similar processes as in other species, yet which are somehow different. These changes were predominantly linked to increased secretory activity associated with preparation for implantation, rather than to proliferative activity. Functional terms associated with ECM remodeling appeared to determine primary functions of canine embryos during the early, pre-attachment stage of pregnancy. This was followed by mobilization of the immune system. The expression and function of cytokines in the species-specific process of embryo maternal recognition in the dog, as well as the intersection between these two systems (i.e., ECM and the immune system), deserve more attention because it can reveal new mechanisms involved in the processes of decidualization and initiation of placentation in dogs. Taking into account the limited invasion of trophoblast during formation of the canine endotheliochorial placenta, such newly acquired information could extend our knowledge about the multiple pathways involved in metabolic and immunological regulation of implantation in mammals.

In summary, this study provides a basis for understanding of the uterine milieu required for proper embryo development and, thereby, for successful establishment of canine pregnancy.

Additionally, activation of some highly conserved proteins, such as IDO and AIF1, was found along with a wide range of chemokines, revealing candidate genes such as IPO, NOV, and DIAPH whose function has so far been underinvestigated. Their functions in preparation for implantation and/or decidualization should be investigated. As for the genes that are expressed jointly in different species, the function of PENK and CYP26A appears to be interesting, but both genes have so far not been recognized as candidates involved in common pathways of evolutionarily determined strategies of embryo-maternal communication.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental File 1. List of genes detectable by microarray in the uterus of early pregnant (pre-attachment) and corresponding control bitches without FDR or *P*-value correction.
Supplemental File 2. List of differentially expressed genes (DEG) between early pregnant and corresponding control bitches with 10% FDR (i.e., adjusted P-value < 0.1).
Supplemental File 3. List of core-enriched genes found by GSEA analysis in the pairwise contrasts between genes expressed in canine uterus vs. "other mammals" (i.e., cow, pig, or horse).
Supplemental File 4. Top 2000 genes with positive scores and top 700 with negative scores of the ranked gene list applied for GSEA, which (A) were used for Venn diagram comparisons, and (B) list of canine-specific genes without overlap with other species obtained from the Venn diagrams.
Supplemental File 5. List of genes overlapping in Venn diagrams between different species, using canine gene set as the background (2000 top positive and 700 top negative scores).
Supplemental File 6. Gene abbreviation index.

Acknowledgments

Authors are grateful to Dr Barry Bavister for careful editing of the manuscript. Part of the laboratory work was performed using the logistics at the Center for Clinical Studies, Vetsuisse Faculty, University of Zurich.

Authors' contributions: FRG: involved in developing the concept of the study, experimental design, generating data, analysis and interpretation of data and writing of the manuscript. AG, EK: knowledge transfer, involvement in the laboratory part of the project, tissue processing. SB: knowledge transfer, analysis and critical discussion of the data. SA and ARA: involved in tissue sampling procedures, animal experiments, and knowledge transfer. AB: knowledge transfer, critical discussion of the data, editing of the manuscript.

MPK: designed and supervised the project, was involved in interpretation of the data, drafting and revising the manuscript.

Conflict of Interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. All authors read and approved the final version of the manuscript.

References

1. Paria BC, Reese J, Das SK, Dey SK. Deciphering the cross-talk of implantation: advances and challenges. *Science* 2002; **296**:2185–2188.
2. Bazer FW. History of maternal recognition of pregnancy. *Adv Anat Embryol Cell Biol* 2015; **216**:5–25.
3. Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, Bayless K. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Mol Hum Reprod* 2010; **16**:135–152.
4. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* 2000; **80**:1–29.
5. Short RV. Implantation and the maternal recognition of pregnancy. In: O'Connor M, Wolstenholme GEW (eds.), *Ciba Foundation Symposium - Foetal Autonomy*. Churchill, London: John Wiley & Sons, Ltd.; 1969: 2–31.
6. Perry JS, Heap RB, Amoroso EC. Steroid hormone production by pig blastocysts. *Nature* 1973; **245**:45–47.
7. Bazer FW, Thatcher WW, Hansen PJ, Mirando MA, Ott TL, Plante C. Physiological mechanisms of pregnancy recognition in ruminants. *J Reprod Fertil Suppl* 1991; **43**:39–47.
8. Spencer TE, Bazer FW. Conceptus signals for establishment and maintenance of pregnancy. *Reprod Biol Endocrinol* 2004; **2**:49.
9. Guillomot M, Flechon JE, Wintenberger-Torres S. Conceptus attachment in the ewe: an ultrastructural study. *Placenta* 1981; **2**:169–182.
10. Boshier DP. A histological and histochemical examination of implantation and early placentome formation in sheep. *J Reprod Fertil* 1969; **19**:51–61.
11. Guillomot M, Guay P. Ultrastructural features of the cell surfaces of uterine and trophoblastic epithelia during embryo attachment in the cow. *Anat Rec* 1982; **204**:315–322.
12. Allen WR, Hamilton DW, Moor RM. The origin of equine endometrial cups. II. Invasion of the endometrium by trophoblast. *Anat Rec* 1973; **177**:485–501.
13. Hoffmann B, Hoveler R, Hasan SH, Failing K. Ovarian and pituitary function in dogs after hysterectomy. *J Reprod Fertil* 1992; **96**:837–845.
14. Kowalewski MP, Schuler G, Taubert A, Engel E, Hoffmann B. Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. *Theriogenology* 2006; **66**:1423–1430.
15. Kowalewski MP, Beceriklisoy HB, Aslan S, Agaoglu AR, Hoffmann B. Time related changes in luteal prostaglandin synthesis and steroidogenic capacity during pregnancy, normal and antiprogestin induced luteolysis in the bitch. *Anim Reprod Sci* 2009; **116**:129–138.
16. Gram A, Buchler U, Boos A, Hoffmann B, Kowalewski MP. Biosynthesis and degradation of canine placental prostaglandins: prepartum changes in expression and function of prostaglandin F2alpha-synthase (PGFS, AKR1C3) and 15-hydroxyprostaglandin dehydrogenase (HPGD). *Biol Reprod* 2013; **89**:2.
17. Concannon PW, McCann JP, Temple M. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *J Reprod Fertil Suppl* 1989; **39**:3–25.
18. Concannon PW. Reproductive cycles of the domestic bitch. *Anim Reprod Sci* 2011; **124**:200–210.
19. Hoffmann B, Hoveler R, Nohr B, Hasan SH. Investigations on hormonal changes around parturition in the dog and the occurrence of pregnancy-specific non conjugated oestrogens. *Exp Clin Endocrinol* 1994; **102**:185–189.
20. Onclin K, Murphy B, Verstegen JP. Comparisons of estradiol, LH and FSH patterns in pregnant and non-pregnant beagle bitches. *Theriogenology* 2002; **57**:1957–1972.
21. Eley RM, Thatcher WW, Bazer FW. Hormonal and physical changes associated with bovine conceptus development. *J Reprod Fertil* 1979; **55**:181–190.
22. Robertson HA, King GJ. Conjugated and unconjugated oestrogens in fetal and maternal fluids of the cow throughout pregnancy. *J Reprod Fertil* 1979; **55**:463–470.
23. Pashen RL, Sheldrick EL, Allen WR, Flint AP. Dehydroepiandrosterone synthesis by the fetal foal and its importance as an oestrogen precursor. *J Reprod Fertil Suppl* 1982; **32**:389–397.
24. Kautz E, Gram A, Aslan S, Ay SS, Selcuk M, Kanca H, Koldas E, Akal E, Karakas K, Findik M, Boos A, Kowalewski MP. Expression of genes involved in the embryo-maternal interaction in the early-pregnant canine uterus. *Reproduction* 2014; **147**:703–717.
25. Graubner FR, Reichler IM, Rahman NA, Payan-Carreira R, Boos A, Kowalewski MP. Decidualization of the canine uterus: From early until late gestational in vivo morphological observations, and functional characterization of immortalized canine uterine stromal cell lines. *Reprod Domest Anim* 2017; **52**(Suppl 2):137–147.
26. Kowalewski MP, Gram A, Kautz E, Graubner FR. The dog: nonconformist, not only in maternal recognition signaling. *Adv Anat Embryol Cell Biol* 2015; **216**:215–237.
27. Vermeirsch H, Simoens P, Lauwers H. Immunohistochemical detection of the estrogen receptor-alpha and progesterone receptor in the canine pregnant uterus and placental labyrinth. *Anat Rec* 2000; **260**:42–50.
28. Kowalewski MP, Beceriklisoy HB, Pfarrer C, Aslan S, Kindahl H, Kucukaslan I, Hoffmann B. Canine placenta: a source of prepartal prostaglandins during normal and antiprogestin-induced parturition. *Reproduction* 2010; **139**:655–664.
29. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 2002; **18**(Suppl 1):S96–S104.
30. Wettenhall JM, Smyth GK. limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics* 2004; **20**: 3705–3706.
31. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for annotation, visualization, and integrated discovery. *Genome Biol* 2003; **4**:P3.
32. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003; **13**:2498–2504.
33. Juengel JL. *Reproduction in Domestic Ruminants VIII: Proceedings of the Ninth International Symposium on Reproduction in Domestic Ruminants, Obihiro, Hokkaido, Japan, August 2014*. Juengel JL, Miyamoto A, Price C, Reynolds LP, Smith MF, Webb R (eds.), Ashby-de-la-Zouch, England: Context Products Ltd.; 2014.
34. Samborski A, Graf A, Krebs S, Kessler B, Reichenbach M, Reichenbach HD, Ulbrich SE, Bauersachs S. Transcriptome changes in the porcine endometrium during the pre-attachment phase. *Biol Reprod* 2013; **89**:134.
35. Merkl M, Ulbrich SE, Otzdorff C, Herbach N, Wanke R, Wolf E, Handler J, Bauersachs S. Microarray analysis of equine endometrium at days 8 and 12 of pregnancy. *Biol Reprod* 2010; **83**:874–886.
36. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci* 2005; **102**:15545–15550.
37. Oliveros JC. Venny. An interactive tool for comparing lists with Venn Diagrams. Available at: http://bioinfogp.cnb.csic.es/tools/venny/index.html; (2207-2015).
38. Altmae S, Reimand J, Hovatta O, Zhang P, Kere J, Laisk T, Saare M, Peters M, Vilo J, Stavreus-Evers A, Salumets A. Research resource: interactome of human embryo implantation: identification of gene expression pathways, regulation, and integrated regulatory networks. *Mol Endocrinol* 2012; **26**:203–217.

39. Kautz E, de Carvalho Papa P, Reichler IM, Gram A, Boos A, Kowalewski MP. In vitro decidualisation of canine uterine stromal cells. *Reprod Biol Endocrinol* 2015; **13**:85.
40. Kowalewski MP, Meyer A, Hoffmann B, Aslan S, Boos A. Expression and functional implications of peroxisome proliferator-activated receptor gamma (PPARgamma) in canine reproductive tissues during normal pregnancy and parturition and at antiprogestin induced abortion. *Theriogenology* 2011; **75**:877–886.
41. Holst PA, Phemister RD. The prenatal development of the dog: preimplantation events. *Biol Reprod* 1971; **5**:194–206.
42. Concannon PW. Endocrinologic control of normal canine ovarian function. *Reprod Domest Anim* 2009; **44**(Suppl 2):3–15.
43. Kehrer A. Chorionic development and structure of the placenta zonaria in the cat, dog and fox (author's transl). *Z Anat Entwicklungsgesch* 1973; **143**:25–42.
44. Amoroso EC. Placentation. In: Parkes AS (ed.), *Marshall's Physiology of Reproduction*. London: Longmans Green; 1952:127–316.
45. Plow EF, Haas TA, Zhang L, Loftus J, Smith JW. Ligand binding to integrins. *J Biol Chem* 2000; **275**:21785–21788.
46. Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, Bilker W, Strom BL. Aberrant integrin expression in the endometrium of women with endometriosis. *J Clin Endocrinol Metab* 1994; **79**:643–649.
47. Bukowska D, Kempisty B, Jackowska M, Wozna M, Antosik P, Piotrowska H, Jaskowski JM. Analysis of integrins and vascular endothelial growth factor isoforms mRNA expression in the canine uterus during perimplantation period. *Pol J Vet Sci* 2011; **14**:253–258.
48. Engvall E, Earwicker D, Haaparanta T, Ruoslahti E, Sanes JR. Distribution and isolation of four laminin variants; tissue restricted distribution of heterotrimers assembled from five different subunits. *Cell Regul* 1990; **1**:731–740.
49. Aplin JD, Charlton AK, Ayad S. An immunohistochemical study of human endometrial extracellular matrix during the menstrual cycle and first trimester of pregnancy. *Cell Tissue Res* 1988; **253**:231–240.
50. Haouzi D, Dechaud H, Assou S, Monzo C, de Vos J, Hamamah S. Transcriptome analysis reveals dialogues between human trophectoderm and endometrial cells during the implantation period. *Hum Reprod* 2011; **26**:1440–1449.
51. Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI et al. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res* 2017; **45**:D200–D203.
52. Mularoni A, Mahfoudi A, Beck L, Coosemans V, Bride J, Nicollier M, Adessi GL. Progesterone control of fibronectin secretion in guinea pig endometrium. *Endocrinology* 1992; **131**:2127–2132.
53. Grinnell F, Head JR, Hoffpauir J. Fibronectin and cell shape in vivo: studies on the endometrium during pregnancy. *J Cell Biol* 1982; **94**:597–606.
54. Bischof P, Meisser A, Campana A. Paracrine and autocrine regulators of trophoblast invasion–a review. *Placenta* 2000; **21**(Suppl A):S55–S60.
55. Jovanovic M, Stefanoska I, Radojcic L, Vicovac L. Interleukin-8 (CXCL8) stimulates trophoblast cell migration and invasion by increasing levels of matrix metalloproteinase (MMP)2 and MMP9 and integrins alpha5 and beta1. *Reproduction* 2010; **139**:789–798.
56. Beceriklisoy HB, Walter I, Schafer-Somi S, Miller I, Kanca H, Izgur H, Aslan S. Matrix metalloproteinase (MMP)-2 and MMP-9 activity in the canine uterus before and during placentation. *Reprod Domest Anim* 2007; **42**:654–659.
57. Schafer-Somi S, Beceriklisoy HB, Budik S, Kanca H, Aksoy OA, Polat B, Cetin Y, Ay SS, Aslan S. Expression of genes in the canine preimplantation uterus and embryo: implications for an active role of the embryo before and during invasion. *Reprod Domest Anim* 2008; **43**:656–663.
58. Imada K, Ito A, Itoh Y, Nagase H, Mori Y. Progesterone increases the production of tissue inhibitor of metalloproteinases-2 in rabbit uterine cervical fibroblasts. *FEBS Lett* 1994; **341**:109–112.
59. Jo YS, Lee GS, Nam SY, Kim SJ. Progesterone Inhibits Leptin-Induced Invasiveness of BeWo Cells. *Int J Med Sci* 2015; **12**:773–779.
60. Monget P, Oxvig C. PAPP-A and the IGF system. *Ann Endocrinol (Paris)* 2016; **77**:90–96.
61. Burnol AF, Morzyglod L, Popineau L. Cross-talk between insulin signaling and cell proliferation pathways. *Ann Endocrinol (Paris)* 2013; **74**:74–78.
62. Jeong W, Song G, Bazer FW, Kim J. Insulin-like growth factor I induces proliferation and migration of porcine trophectoderm cells through multiple cell signaling pathways, including protooncogenic protein kinase 1 and mitogen-activated protein kinase. *Mol Cell Endocrinol* 2014; **384**:175–184.
63. Li SW, Li L, Ma Y, Cao ZY, Peng ZL, Han ZY. The effects of progesterone and insulin-like growth factor (IGF- I and IGF-II) on proliferation in human decidual stromal cells of early pregnancy in vitro. *Sichuan Da Xue Xue Bao Yi Xue Ban* 2009; **40**:442–444.
64. Kashiwagi A, DiGirolamo CM, Kanda Y, Niikura Y, Esmon CT, Hansen TR, Shioda T, Pru JK. The postimplantation embryo differentially regulates endometrial gene expression and decidualization. *Endocrinology* 2007; **148**:4173–4184.
65. Pringle KG, Roberts CT. New light on early post-implantation pregnancy in the mouse: roles for insulin-like growth factor-II (IGF-II)? *Placenta* 2007; **28**:286–297.
66. Fagotto F, Gluck U, Gumbiner BM. Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. *Curr Biol* 1998; **8**:181–190.
67. Jakel S, Mingot JM, Schwarzmaier P, Hartmann E, Gorlich D. Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. *Embo J* 2002; **21**:377–386.
68. Sonderegger S, Pollheimer J, Knofler M. Wnt signalling in implantation, decidualisation and placental differentiation–review. *Placenta* 2010; **31**:839–847.
69. Matsumiya T, Xing F, Ebina M, Hayakari R, Imaizumi T, Yoshida H, Kikuchi H, Topham MK, Satoh K, Stafforini DM. Novel role for molecular transporter importin 9 in posttranscriptional regulation of IFN-epsilon expression. *J Immunol* 2013; **191**:1907–1915.
70. Hardy MP, Owczarek CM, Jermiin LS, Ejdeback M, Hertzog PJ. Characterization of the type I interferon locus and identification of novel genes. *Genomics* 2004; **84**:331–345.
71. Moriyama T, Nagai M, Oka M, Ikawa M, Okabe M, Yoneda Y. Targeted disruption of one of the importin alpha family members leads to female functional incompetence in delivery. *Febs j* 2011; **278**:1561–1572.
72. Xu K, Tao T, Jie J, Lu X, Li X, Mehmood MA, He H, Liu Z, Xiao X, Yang J, Ma JX, Li W et al. Increased importin 13 activity is associated with the pathogenesis of pterygium. *Mol Vis* 2013; **19**:604–613.
73. Kimura M, Imamoto N. Biological significance of the importin-beta family-dependent nucleocytoplasmic transport pathways. *Traffic* 2014; **15**:727–748.
74. Huang L, Yoneda M, Kimata K. A serum-derived hyaluronan-associated protein (SHAP) is the heavy chain of the inter alpha-trypsin inhibitor. *J Biol Chem* 1993; **268**:26725–26730.
75. Gonzalez-Ramon N, Alava MA, Sarsa JA, Pineiro M, Escartin A, Garcia-Gil A, Lampreave F, Pineiro A. The major acute phase serum protein in pigs is homologous to human plasma kallikrein sensitive PK-120. *FEBS Lett* 1995; **371**:227–230.
76. Geisert RD, Ashworth MD, Malayer JR. Expression of inter-alpha-trypsin inhibitor heavy chains in endometrium of cyclic and pregnant gilts. *Reproduction* 2003; **126**:621–627.
77. Choi-Miura NH, Takahashi K, Yoda M, Saito K, Hori M, Ozaki H, Mazda T, Tomita M. The novel acute phase protein, IHRP, inhibits actin polymerization and phagocytosis of polymorphonuclear cells. *Inflamm Res* 2000; **49**:305–310.
78. Jun JI, Lau LF. Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. *Nat Rev Drug Discov* 2011; **10**:945–963.
79. Holbourn KP, Acharya KR, Perbal B. The CCN family of proteins: structure-function relationships. *Trends Biochem Sci* 2008; **33**:461–473.
80. Winterhager E, Gellhaus A. The role of the CCN family of proteins in female reproduction. *Cell Mol Life Sci* 2014; **71**:2299–2311.

81. Ishihara J, Umemoto T, Yamato M, Shiratsuchi Y, Takaki S, Petrich BG, Nakauchi H, Eto K, Kitamura T, Okano T. Nov/CCN3 regulates long-term repopulating activity of murine hematopoietic stem cells via integrin alphavbeta3. *Int J Hematol* 2014; **99**:393–406.
82. Gellhaus A, Schmidt M, Dunk C, Lye SJ, Kimmig R, Winterhager E. Decreased expression of the angiogenic regulators CYR61 (CCN1) and NOV (CCN3) in human placenta is associated with pre-eclampsia. *Mol Hum Reprod* 2006; **12**:389–399.
83. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000; **182**:311–322.
84. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984; **133**:1710–1715.
85. Rahmanzadeh R, Huttmann G, Gerdes J, Scholzen T. Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis. *Cell Prolif* 2007; **40**:422–430.
86. Ji P, Jayapal SR, Lodish HF. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. *Nat Cell Biol* 2008; **10**:314–321.
87. Goode BL, Eck MJ. Mechanism and function of formins in the control of actin assembly. *Annu Rev Biochem* 2007; **76**:593–627.
88. Beli P, Mascheroni D, Xu D, Innocenti M. WAVE and Arp2/3 jointly inhibit filopodium formation by entering into a complex with mDia2. *Nat Cell Biol* 2008; **10**:849–857.
89. Young LE, Heimsath EG, Higgs HN. Cell type-dependent mechanisms for formin-mediated assembly of filopodia. *Mol Biol Cell* 2015; **26**:4646–4659.
90. Yang C, Czech L, Gerboth S, Kojima S, Scita G, Svitkina T. Novel roles of formin mDia2 in lamellipodia and filopodia formation in motile cells. *PLoS Biol* 2007; **5**:e317.
91. Wallar BJ, Deward AD, Resau JH, Alberts AS. RhoB and the mammalian Diaphanous-related formin mDia2 in endosome trafficking. *Exp Cell Res* 2007; **313**:560–571.
92. Watanabe S, Ando Y, Yasuda S, Hosoya H, Watanabe N, Ishizaki T, Narumiya S. mDia2 induces the actin scaffold for the contractile ring and stabilizes its position during cytokinesis in NIH 3T3 cells. *Mol Biol Cell* 2008; **19**:2328–2338.
93. Eisenmann KM, Harris ES, Kitchen SM, Holman HA, Higgs HN, Alberts AS. Dia-interacting protein modulates formin-mediated actin assembly at the cell cortex. *Curr Biol* 2007; **17**:579–591.
94. Lizarraga F, Poincloux R, Romao M, Montagnac G, Le Dez, G, Bonne, I, Rigaill G, Raposo G, Chavrier P. Diaphanous-related formins are required for invadopodia formation and invasion of breast tumor cells. *Cancer Res* 2009; **69**:2792–2800.
95. Daou P, Hasan S, Breitsprecher D, Baudelet E, Camoin L, Audebert S, Goode BL, Badache A. Essential and nonredundant roles for Diaphanous formins in cortical microtubule capture and directed cell migration. *Mol Biol Cell* 2014; **25**:658–668.
96. Chesarone MA, DuPage AG, Goode BL. Unleashing formins to remodel the actin and microtubule cytoskeletons. *Nat Rev Mol Cell Biol* 2010; **11**:62–74.
97. Narumiya S, Tanji M, Ishizaki T. Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. *Cancer Metastasis Rev* 2009; **28**:65–76.
98. Di Vizio D, Kim J, Hager MH, Morello M, Yang W, Lafargue CJ, True LD, Rubin MA, Adam RM, Beroukhim R, Demichelis F, Freeman MR. Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease. *Cancer Res* 2009; **69**:5601–5609.
99. Evans J, Anderton D. Pregnancy diagnosis in the bitch: the development of a test based on the measurement of acute phase proteins in the blood. *Annales De Zootechnie* 1992; **41**:397–405.
100. Concannon PW, Gimpel T, Newton L, Castracane VD. Postimplantation increase in plasma fibrinogen concentration with increase in relaxin concentration in pregnant dogs. *Am J Vet Res* 1996; **57**:1382–1385.
101. Eckersall PD, Harvey MJ, Ferguson JM, Renton JP, Nickson DA, Boyd JS. Acute phase proteins in canine pregnancy (Canis familiaris). *J Reprod Fertil Suppl* 1993; **47**:159–164.
102. Thomas T, Fletcher S, Yeoh GC, Schreiber G. The expression of alpha(1)-acid glycoprotein mRNA during rat development. High levels of expression in the decidua. *J Biol Chem* 1989; **264**:5784–5790.
103. Hunt JS. Cytokine networks in the uteroplacental unit: macrophages as pivotal regulatory cells. *J Reprod Immunol* 1989; **16**:1–17.
104. Ben-Hur H, Gurevich P, Berman V, Tchanyshev R, Gurevich E, Zusman I. The secretory immune system as part of the placental barrier in the second trimester of pregnancy in humans. *In Vivo* 2001; **15**:429–435.
105. Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G. Macrophages and apoptotic cell clearance during pregnancy. *Am J Reprod Immunol* 2004; **51**:275–282.
106. Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA. Mucins in the mucosal barrier to infection. *Mucosal Immunol* 2008; **1**:183–197.
107. King AE, Critchley HO, Kelly RW. Innate immune defences in the human endometrium. *Reprod Biol Endocrinol* 2003; **1**:116.
108. Roberts RM, Bazer FW, Baldwin N, Pollard WE. Progesterone induction of lysozyme and peptidase activities in the porcine uterus. *Arch Biochem Biophys* 1976; **177**:499–507.
109. Miwa N, Hayakawa S, Miyazaki S, Myojo S, Sasaki Y, Sakai M, Takikawa O, Saito S. IDO expression on decidual and peripheral blood dendritic cells and monocytes/macrophages after treatment with CTLA-4 or interferon-gamma increase in normal pregnancy but decrease in spontaneous abortion. *Mol Hum Reprod* 2005; **11**:865–870.
110. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J Exp Med* 1999; **189**:1363–1372.
111. Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J Immunol* 2000; **164**:3596–3599.
112. Yan Y, Zhang G-X, Gran B, Fallarino F, Yu S, Li H, Cullimore ML, Rostami A, Xu H. IDO upregulates regulatory T cells via tryptophan catabolite and suppresses encephalitogenic T cell responses in experimental autoimmune encephalomyelitis. *J Immunol* 2010; **185**:5953–5961.
113. Croxatto D, Vacca P, Canegallo F, Conte R, Venturini PL, Moretta L, Mingari MC. Stromal cells from human decidua exert a strong inhibitory effect on NK cell function and dendritic cell differentiation. *PLoS One* 2014; **9**:e89006.
114. Lobo SC, Huang ST, Germeyer A, Dosiou C, Vo KC, Tulac S, Nayak NR, Giudice LC. The immune environment in human endometrium during the window of implantation. *Am J Reprod Immunol* 2004; **52**:244–251.
115. Gutierrez G, Fitzgerald JS, Pohlmann T, Hoppe I, Markert UR. Comparative effects of L-tryptophan and 1-methyl-tryptophan on immunoregulation induced by sperm, human pre-implantation embryo and trophoblast supernatants. *Am J Reprod Immunol* 2003; **50**:309–315.
116. King A. Uterine leukocytes and decidualization. *Hum Reprod Update* 2000; **6**:28–36.
117. Bulmer JN, Williams PJ, Lash GE. Immune cells in the placental bed. *Int J Dev Biol* 2010; **54**:281–294.
118. Moffett A, Loke C. Immunology of placentation in eutherian mammals. *Nat Rev Immunol* 2006; **6**:584–594.
119. Zhao YY, Yan DJ, Chen ZW. Role of AIF-1 in the regulation of inflammatory activation and diverse disease processes. *Cell Immunol* 2013; **284**:75–83.
120. Liu G, Ma H, Jiang L, Zhao Y. Allograft inflammatory factor-1 and its immune regulation. *Autoimmunity* 2007; **40**:95–102.
121. Chen X, Kelemen SE, Autieri MV. AIF-1 expression modulates proliferation of human vascular smooth muscle cells by autocrine expression of G-CSF. *Arterioscler Thromb Vasc Biol* 2004; **24**:1217–1222.
122. Jia J, Cai Y, Wang R, Fu K, Zhao YF. Overexpression of allograft inflammatory factor-1 promotes the proliferation and migration of human endothelial cells (HUV-EC-C) probably by up-regulation of basic fibroblast growth factor. *Pediatr Res* 2010; **67**:29–34.
123. Tian Y, Jain S, Kelemen SE, Autieri MV. AIF-1 expression regulates endothelial cell activation, signal transduction, and vasculogenesis. *Am J Physiol Cell Physiol* 2009; **296**:C256–C266.
124. Utans U, Arceci RJ, Yamashita Y, Russell ME. Cloning and characterization of allograft inflammatory factor-1: a novel macrophage factor

identified in rat cardiac allografts with chronic rejection. *J Clin Invest* 1995; **95**:2954–2962.
125. Shimada S, Iwabuchi K, Watano K, Shimizu H, Yamada H, Minakami H, Onoe K. Expression of allograft inflammatory factor-1 in mouse uterus and poly(I:C)-induced fetal resorption. *Am J Reprod Immunol* 2003; **50**:104–112.
126. Sharkey A. Cytokines and implantation. *Rev Reprod* 1998; **3**:52–61.
127. Tartakovsky B, Ben-Yair E. Cytokines modulate pre-implantation development and pregnancy. *Dev Biol* 1991; **146**:345–352.
128. Simón C, Moreno C, Remohí J, Pellicer A. Cytokines and embryo implantation1. *J Reprod Immunol* 1998; **39**:117–131.
129. Zheng H, Fu G, Dai T, Huang H. Migration of endothelial progenitor cells mediated by stromal cell-derived factor-1alpha/CXCR4 via PI3K/Akt/eNOS signal transduction pathway. *J Cardiovasc Pharmacol* 2007; **50**:274–280.
130. Matloubian M, David A, Engel S, Ryan JE, Cyster JG. A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat Immunol* 2000; **1**:298–304.
131. Huang Y, Li DJ, Wang MY, Cheng HD. [Expressions of CXCL16/CXCR6 and CXCL12/CXCR4 in first-trimester human trophoblast cells]. *Zhonghua Fu Chan Ke Za Zhi* 2006; **41**:399–402.
132. Huang Y, Zhu XY, Du MR, Wu X, Wang MY, Li DJ. Chemokine CXCL16, a scavenger receptor, induces proliferation and invasion of first-trimester human trophoblast cells in an autocrine manner. *Hum Reprod* 2006; **21**:1083–1091.
133. Schanz A, Baston-Bust D, Krussel JS, Heiss C, Janni W, Hess AP. CXCR7 and syndecan-4 are potential receptors for CXCL12 in human cytotrophoblasts. *J Reprod Immunol* 2011; **89**:18–25.
134. Rees MC, Kelly RW. Prostaglandin D2 release by endometrium and myometrium. *Br J Obstet Gynaecol* 1986; **93**:1078–1082.
135. Norwitz ER, Starkey PM, Lopez Bernal A. Prostaglandin D2 production by term human decidua: cellular origins defined using flow cytometry. *Obstet Gynecol* 1992; **80**:440–445.
136. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takano S, Nagata K. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* 2001; **193**:255–261.
137. Michimata T, Tsuda H, Sakai M, Fujimura M, Nagata K, Nakamura M, Saito S. Accumulation of CRTH2-positive T-helper 2 and T-cytotoxic 2 cells at implantation sites of human decidua in a prostaglandin D(2)-mediated manner. *Mol Hum Reprod* 2002; **8**:181–187.
138. Saito S, Tsuda H, Michimata T. Prostaglandin D2 and reproduction. *Am J Reprod Immunol* 2002; **47**:295–302.
139. Piraino G, Cook JA, O'Connor M, Hake PW, Burroughs TJ, Teti D, Zingarelli B. Synergistic effect of peroxisome proliferator activated receptor-gamma and liver X receptor-alpha in the regulation of inflammation in macrophages. *Shock* 2006; **26**:146–153.
140. Jones JR, Barrick C, Kim KA, Lindner J, Blondeau B, Fujimoto Y, Shiota M, Kesterson RA, Kahn BB, Magnuson MA. Deletion of PPARgamma in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance. *Proc Natl Acad Sci U S A* 2005; **102**:6207–6212.
141. Li Y, Qi Y, Huang TH, Yamahara J, Roufogalis BD. Pomegranate flower: a unique traditional antidiabetic medicine with dual PPAR-alpha/-gamma activator properties. *Diabetes Obes Metab* 2008; **10**:10–17.
142. Ackerman WET, Zhang XL, Rovin BH, Kniss DA. Modulation of cytokine-induced cyclooxygenase 2 expression by PPARG ligands through NFkappaB signal disruption in human WISH and amnion cells. *Biol Reprod* 2005; **73**:527–535.
143. Chen LR, Lee SC, Lin YP, Hsieh YL, Chen YL, Yang JR, Liou JF, Chen CF, Lee YP, Shiue YL. Prostaglandin-D synthetase induces transcription of the LH beta subunit in the primary culture of chicken anterior pituitary cells via the PPAR signaling pathway. *Theriogenology* 2010; **73**:367–382.
144. Bazer FW. Establishment of pregnancy in sheep and pigs. *Reprod Fertil Dev* 1989; **1**:237–242.
145. Concannon PW. Endocrinologic control of normal canine ovarian function. *Reprod Domest Anim* 2009; **44**(Suppl 2):3–15.
146. Concannon P, Tsutsui T, Shille V. Embryo development, hormonal requirements and maternal responses during canine pregnancy. *J Reprod Fertil Suppl* 2001; **57**:169–179.
147. Su RW, Fazleabas AT. Implantation and Establishment of Pregnancy in Human and Nonhuman Primates. *Adv Anat Embryol Cell Biol* 2015; **216**:189–213.
148. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med* 2007; **25**:445–453.
149. Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocr Rev* 2014; **35**:851–905.
150. Jin DF, Muffly KE, Okulicz WC, Kilpatrick DL. Estrous cycle- and pregnancy-related differences in expression of the proenkephalin and proopiomelanocortin genes in the ovary and uterus. *Endocrinology* 1988; **122**:1466–1471.
151. Zhu Y, Pintar JE. Expression of opioid receptors and ligands in pregnant mouse uterus and placenta. *Biol Reprod* 1998; **59**:925–932.
152. Han BC, Xia HF, Sun J, Yang Y, Peng JP. Retinoic acid-metabolizing enzyme cytochrome P450 26a1 (cyp26a1) is essential for implantation: functional study of its role in early pregnancy. *J Cell Physiol* 2010; **223**:471–479.
153. Xia HF, Ma JJ, Sun J, Yang Y, Peng JP. Retinoic acid metabolizing enzyme CYP26A1 is implicated in rat embryo implantation. *Hum Reprod* 2010; **25**:2985–2998.

Manuscript 2: Uterine and placental distribution of selected ECM components in the dog.

Graubner FR, Boos A, Aslan S, Kücükaslan I, Kowalewski MP.

Published in: Reproduction. 2018 May 1;155(5):403-421 doi: 10.1530/REP-17-0761. Epub 2018 Feb 9. PMID: 29439094

Contribution:
Felix R. Graubner was involved in developing the concept of the study, experimental design, generating data, analysis and interpretation of data and writing of the manuscript.

REPRODUCTION
RESEARCH

Uterine and placental distribution of selected extracellular matrix (ECM) components in the dog

Felix R Graubner[1], Alois Boos[1], Selim Aslan[2], Ibrahim Kücükaslan[3] and Mariusz P Kowalewski[1]

[1]*Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland,* [2]*Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Near East University, Nicosia, North Cyprus, Turkey and* [3]*Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Dicle University, Diyarbakir, Turkey*

Correspondence should be addressed to M P Kowalewski; Email: kowalewski@vetanat.uzh.ch

Abstract

For many years, modifications of the uterine extracellular matrix (ECM) during gestation have not been considered as critical for successful canine (*Canis lupus familiaris*) pregnancy. However, previous reports indicated an effect of free-floating blastocysts on the composition of the uterine ECM. Here, the expression of selected genes involved in structural functions, cell-to-cell communication and inhibition of matrix metalloproteinases were targeted utilizing qPCR and immunohistochemistry. We found that canine free-floating embryos affect gene expression of *FN1*, *ECM1* and *TIMP4*. This seems to be associated with modulation of trophoblast invasion, and proliferative and adhesive functions of the uterus. Although not modulated at the beginning of pregnancy, the decrease of structural ECM components (i.e. *COL1*, *-3*, *-4* and *LAMA2*) from pre-implantation toward post-implantation at placentation sites appears to be associated with softening of the tissue in preparation for trophoblast invasion. The further decrease of these components at placentation sites at the time of prepartum luteolysis seems to be associated with preparation for the release of fetal membranes. Reflecting a high degree of communication, intercellular cell adhesion molecules are induced following placentation (*Cx26*) or increase gradually toward prepartum luteolysis (*Cx43*). The spatio-temporal expression of TIMPs suggests their active involvement in modulating fetal invasiveness, and together with *ECM1*, they appear to protect deeper endometrial structures from trophoblast invasion. With this, the dog appears to be an interesting model for investigating placental functions in other species, e.g. in humans in which *Placenta accreta* appears to share several similarities with canine subinvolution of placental sites (SIPS). In summary, the canine uterine ECM is only moderately modified in early pregnancy, but undergoes vigorous reorganization processes in the uterus and placenta following implantation.

Reproduction (2018) **155** 403–421

Introduction

Interactions between the canine (*Canis lupus familiaris*) uterus and the corpora lutea (CL), and the interchange between the uterus and fetal trophoblast, represent remarkably unique mechanisms among mammalian species.

The bitch is a monoestric, non-seasonal breeder in which ovulation occurs spontaneously (Concannon 1993). As in other domestic animals, CL are formed following ovulation, but in contrast to other species, in canine pregnancy, no placental steroid hormone synthesis arises (Hoffmann *et al.* 1994). Consequently, the increasing circulating steroid levels in both non-pregnant and pregnant bitches fully originate from the CL with luteal progesterone (P4) being responsible for the establishment and maintenance of pregnancy until term (Concannon *et al.* 1989). Due to the lack of luteolysis in the absence of pregnancy, peripheral P4 levels are almost identical in non-pregnant and pregnant bitches until shortly before parturition when prepartum luteolysis occurs signaling the onset of parturition (Concannon *et al.* 1989). Consequently, in contrast to livestock, in the dog, successful pregnancy does not depend on the inhibition of luteolysis to maintain the luteal life span and thereby embryo survival (Kowalewski *et al.* 2009, Gram *et al.* 2013, Kowalewski 2014). In other domestic animal species, pre-attachment embryos transmit signals needed to extend CL function (Niswender *et al.* 2000, Bazer *et al.* 2010, Bazer 2015), referred to as 'maternal recognition of pregnancy' (Short 1969). This embryo–maternal communication synchronizes blastocyst development and uterine receptivity in a complex series of molecular and cellular mechanisms (Seshagiri *et al.* 2009, Paidas *et al.* 2010). In some species, e.g.- in rodents or primates, the uterine changes result in decidua formation and are therefore very pronounced. In primates, decidualization takes place even in the absence of embryos, while in rodents, it is induced by their presence (reviewed in Cha *et al.* 2012). In early pregnancy, the uterine extracellular matrix (ECM) influences trophoblast invasion (Johnson *et al.*

ISSN 1470–1626 (paper) 1741–7899 (online)
https://doi.org/10.1530/REP-17-0761
Online version via www.reproduction-online.org

2003) and remodeling of the decidua (Damsky *et al.* 1993, Lala & Nandi 2016, Smith *et al.* 2016). The formation of decidua, e.g.- in mice, is associated with clearing of hyaluronic acid from the uterine stroma (Brown & Papaioannou 1992). In addition, *in vitro* studies demonstrate that decidualizing stromal cells of mouse and human origin produce ECM products such as basal lamina-like materials (Wewer *et al.* 1985, 1986, Kisalus *et al.* 1987).

Despite the absence of an antiluteolytic signal at the beginning of pregnancy, in the dog synchronization between blastocyst development and uterine preparation for implantation is also essential for healthy pregnancy. Thus, in dogs and other carnivores, decidualization also takes place and is associated with implantation and placenta formation (Wislocki & Dempsey 1946, Dempsey & Wislocki 1956, Kautz *et al.* 2014, Graubner *et al.* 2017*b*). In accordance with the aforementioned *in vitro* decidualization studies with mouse and human cells, canine uterine cells can also be decidualized *in vitro*. Their response to a decidual stimulus (i.e.- increased secretory and proliferative activity) provides evidence that also in dogs uterine ECM modulation during decidualization occurs (Kautz *et al.* 2015). Furthermore, functional genomics studies imply that *in vivo* canine free-floating embryos transmit signals to the uterus and discretely, i.e., without clearly visible morphological changes, affect uterine ECM composition at the early stage of pregnancy (Graubner *et al.* 2017*a*). Indicating the beginning of canine decidualization, these embryo-derived signals control the expression of uterine biochemical markers of decidualization (Kautz *et al.* 2014). The first morphological signs of decidualization are found later, commencing with attachment of the trophoblast (Graubner *et al.* 2017*b*). Ultimately, the uterine modifications become advanced and maternal-derived decidual cells are formed as an indispensable component of the endotheliochorial placenta in the dog. Decidual cells are needed for successful pregnancy since they are the only cells in the canine placenta expressing the nuclear P4 receptor (PGR) (Vermeirsch *et al.* 2000, Kowalewski *et al.* 2010). Additionally, these cells and maternal endothelium can resist trophoblast digestion. Adequate embryo–maternal contacts between the trophoblast and maternal decidual cells, all embedded in the ECM components, are important for the maintenance and termination of pregnancy. Thus, withdrawal of P4 from decidual cells will interfere with their physiological function and lead to abortion or preterm parturition (Kowalewski *et al.* 2010). ECM components appear to be involved here in feto-maternal communication leading to the luteolytic cascade, expulsion of fetuses and fetal membranes. Disturbances in embryo–maternal communication may result in clinical conditions like subinvolution of placental sites (SIPS), resembling placenta accreta in humans characterized by excessive trophoblast invasion.

Formation of the endotheliochorial placenta in the dog and other carnivores, indeed represents a unique type of placentation, with strong, but yet restricted, invasion. So far, little attention has been paid to uterine function in dogs during pregnancy, resulting in limited knowledge about its possible involvement in the establishment, maintenance and termination of pregnancy.

Because of this lack of information, driven by the hypothesis that ECM compounds are strongly modified throughout canine gestation, this study focused on the localization (utilizing immunohistochemistry (IHC)) and expression (using semi-quantitative TaqMan PCR) of several ECM proteins in the canine uterus. Selected stages of the reproductive cycle were considered, starting with morpho-functional modifications induced by the presence of pre-attachment embryos, through implantation, early and late placentation, until prepartum luteolysis. A variety of different factors collectively represent the term ECM. Here, the uterine localization and expression of ECM components with structural and adhesive functions were investigated (e.g.- collagen (COL) 1, -3 and -4, alpha smooth muscle actin (aSMA) and fibronectin (FN) 1). Additionally, proteins of the basal lamina, proteins involved in cell-to-cell communication and proteins involved in the inhibition of matrix metalloproteinases were targeted (e.g.- connexin (Cx) 26 and -43, laminin alpha (LAMA) 2, extracellular matrix protein 1 (ECM1) and tissue inhibitor of metalloproteinases (TIMP) 2 and -4).

Materials and methods

Sample collection

In the present study, tissue material from 31 crossbred healthy bitches aged 2–8 years was used. Animals were randomly assigned to the following groups: (1) early pregnant (pre-attachment days 10–12; $n=10$); (2) corresponding non-pregnant controls ($n=8$); (3) early post-implantation (days 18–25 of pregnancy; $n=5$); (4) mid-gestation (days 35–40 of pregnancy; $n=5$) and (5) prepartum luteolysis ($n=3$).

Estrus detection was performed by cytological evaluation of vaginal smears and regular measurements of P4 concentrations every 2–3 days starting with the proestrous bleeding. The day when P4 blood levels increased >5 ng/mL for the first time was determined as the day of ovulation. Considering the peculiar need for canine oocyte maturation within the oviduct (on average 2–3 days after ovulation), bitches were mated 2 days after ovulation (defined as day 0 of gestation). Tissue collection was performed through routine ovariohysterectomy (OHE). In the early pregnant group (group 1), OHE was performed between days 10 and 12 of gestation. Pregnancy was confirmed by flushing free-floating (i.e., pre-attachment) embryos. Dogs negative for embryo flushing were allotted to group 2 (corresponding non-pregnant control at day 10–12 of the luteal phase). The prepartum luteolysis takes place in the dog 12–24 h before any visible physiological signs of parturition. Therefore, for group 5, blood samples were collected every 6 h

starting on day 58 of pregnancy. When circulating P4 levels continued to decrease below 2–3 ng/mL in 3 consecutive measurements, indicating the prepartum luteolysis, samples were collected. Tissue collection in groups 1 and 2 consisted of uterine cross-sections (the whole thickness of the uterine wall). For groups 3–5, sampling included utero-placental cross-sections (Ut-Pl; the whole thickness of the uterine wall, i.e., uterus with adjacent placenta). Additionally, for groups 3 and 4 inter-placental uterine cross-sections (inter-Pl; full thickness like groups 1 and 2) were collected. No inter-Pl samples were available from the natural luteolysis group of dogs (group 5).

For semi-quantitative real-time PCR, cellular RNA was stabilized by immersing the samples for 24 h at 4°C in RNAlater (Ambion Biotechnologie GmbH, Wiesbaden, Germany); prolonged storage was at −80°C. For immunohistochemistry (IHC) studies, tissues were fixed for 24 h in 10% neutral phosphate-buffered formalin at 4°C, and subsequently washed for 1 week with PBS (phosphate-buffered saline) and embedded in paraffin wax. For additional information about tissue sampling and processing procedures, see previous reports (Kowalewski *et al.* 2010, Gram *et al.* 2013, Kautz *et al.* 2014). All uterine tissue samples were used for semi-quantitative real-time PCR, while 3 randomly chosen samples from each group were used for IHC.

All experimental procedures were carried out in accordance with animal welfare legislation and approved by the respective authorities of the University of Ankara (permits no. 2006/06 and 2008-25-124) Ankara, Turkey and Justus-Liebig University Giessen (permit no. II 25.3-19c20-15c GI 18/14 and VIG3-19c-20/15c GI 18, 14) Giessen, Germany.

RNA isolation, reverse transcription (RT), semi-quantitative (TaqMan) PCR and evaluation of data

Semi-quantitative TaqMan PCR was performed on total RNA isolated from all dogs. For this, TRIzol reagent (Invitrogen) was used according to the manufacturer's instructions. From each sample 10 ng total RNA were used for RQ1 RNAse-free DNAse treatment (Promega). The RT reaction was performed according to the manufacturer's instructions with the High-Capacity cDNA Reverse Transcription Kit including RNase Inhibitor (Applied Biosystems from Thermo Fisher Scientific). Following this, amplification of cDNA was performed with the TaqManPreAmp Master Mix Kit (Applied Biosystems) according to the supplier's protocol. Detailed information about the TaqMan PCR procedure has been previously published (Kowalewski *et al.* 2006*b*, 2011, Kautz *et al.* 2015). In short, reactions were run in duplicates with the Fast Start Universal Probe Master (ROX) (Roche Diagnostics AG) in an automated fluorometer (ABI PRISM 7500 Sequence Detection System, Applied Biosystems). For negative controls, the so-called RT-minus control was used to check for potential genomic DNA contamination. Additional controls consisted of running experiments with autoclaved water instead of cDNA (no-template control) (Kowalewski *et al.* 2006*b*, 2011). If canine-specific TaqMan systems were not available commercially, Primer Express Software v2.0 (Applied Biosystems) was used to select primers and probes, which were purchased from Microsynth, Balgach, Switzerland. TaqMan probes were designed with 6-FAM- and TAMRA-labels. For a list of self-designed primer sequences and TaqMan systems refer Table 1. The self-designed expression assays were validated by the CT slope method, which tests the TaqMan efficiency at different quantities of the targeted RNA as previously described (Kowalewski *et al.* 2011). The reaction efficiency for all self-designed assays was approximately 100%. Three reference genes, *GAPDH*, *Cyclophilin A* (*PPIA*) and *ACTIN-B*, were used for normalization to ensure homogeneity of variances. The following canine-specific TaqMan Gene Expression Assays were ordered from Applied Biosystems: *PPIA* (Prod. No. Cf03986523-gH); *ACTIN-B* (Prod. No. Cf03023880_g1), collagen type 1, alpha 1 (*COL1A1*) (Prod. No. Cf02741575_mH); COL type 3, alpha 1 (*COL3A1*) (Prod. No. Cf02631366_m1); COL type 4, alpha 1 (*COL4A1*) (Prod. No. Cf02696157_mH). For relative quantification, the comparative CT method (ΔΔCT method) was applied as previously described (Kowalewski *et al.* 2010, 2011). The sample with the lowest expression was used as a calibrator.

Statistical differences between the non-pregnant and early pregnant group (groups 1 and 2) were determined by an unpaired, two-tailed Student's *t*-test; a P value <0.05 was considered as statistically significant. When target gene expression was compared in more than 2 observational groups (time-dependent expression at selected time points during pregnancy), a parametric one-way ANOVA was applied. In the case of $P<0.05$, the Tukey–Kramer multiple comparisons post-test was performed. Statistical assumptions, such as normality and equality of variances were tested prior to applying the t-test or ANOVA. All analyses were performed with GraphPad 3.06 software (GraphPad Software). Numerical data are presented as geometric means Xg ± geometric standard deviation (s.d.).

Immunohistochemistry

Standard immunohistochemistry (IHC) was performed to identify the localization of several ECM proteins. Detailed information about our indirect immunoperoxidase method has been published previously (Kowalewski *et al.* 2006*a*,*b*). In short, following embedding in paraffin, tissues were sectioned into 2–3 µm thickness, mounted onto SuperFrost microscope slides (Menzel-Glaser, Braunschweig, Germany). Following de-paraffinization using xylene and rehydratation in an ethanol series, antigen retrieval was performed using either heat-induced epitope retrieval (HIER) or protease-induced epitope retrieval (PIER). HIER was performed in a microwave oven at 560 W for 15 min in 10 mM citrate buffer with pH 6.0. PIER was performed using 0.25% pepsin (P7000 Sigma-Aldrich Chemie GmbH) in 10 mM HCl at 37°C. The duration of PIER varied from 5 min to 90 min depending on the antibody (for detailed information see Table 2). Following antigen retrieval, sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidases. After that, blocking was performed with 10% normal serum from the same species in which the secondary antibody was produced, and overlaid with primary antibodies overnight at 4°C. For negative controls, non-immune IgGs of the same species instead of the primary

Table 1 List of primers used for real-time (TaqMan) PCR.

Primer	Accession number	Primer sequence			Product length (bp)
		Forward	Reverse	TaqMan Probe	
ECM1	XM_845921.4	5′-CAG TCT GGC TTC TCC CAC CTT A-3′	5′-GCG GTT TGT GTG GCT GTG A-3′	5′-AGA CTA GAT ATT CCC GCT GCC GCT-3′	99
Cx26	AJ439693.1	5′-CCA CTA CTT CCC CAT CTC TCA CA-3′	5′-TCC GGT AGG CGA CAT GCA T-3′	5′-CCG ACT CTG GGC TCT GCA GCT GAT C-3′	98
Cx43	AY462223	5′-AAA AGA GAA CCC TGC CCT CAT C-3′	5′-AGG ACA CGA CCA GCA TGA AGA-3′	5′-ACT GCT TCC TCT CTC GCC CCA CG-3′	91
FN1	XM_014110981	5′-CAC GCC GAA CTA CGA TGC-3′	5′-TGC GAT ACA TGA CCC CTT-3′	5′-AAG TTT GGA TTT TGC CCC ATG GCC-3′	95
LAMA2	XM_014113700.1	5′-AAA CCG GCT CAC GAT TGA G-3′	5′-AGT TGA ACG GTG GCG AAG T-3′	5′-CCT GCT CTT CTA CAT GGC TCG GAT CAA-3′	99
GAPDH	AB028142	5′-GCT GCC AAA TAT GAC GAC ATC A-3′	5′-GTA GCC CAG GAT GCC TTT GAG-3′	5′-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3′	75
TIMP2	AF188489	5′-CAT AGG TAC CAG ATG GGC TGT GA-3′	5′-CAG TCC ATC CAG AGG CAC TCA-3′	5′-TGA TCC CGT GCT ATA TCT CGT CTC CGG-3′	95
TIMP4	NM_001314106	5′-CTG TGG CTG CCA AAT TAC CA-3′	5′-CCC ATA GAG CTT CCG TTC CA-3′	5′-ACC ATC TCA GCC CCT AAC GAG TGC CTC-3′	103

antibody at the same protein concentration as for the primary antibody were used (isotype controls).

Following incubation with the primary antibody, sections were washed with IHC buffer/0.3% Triton X pH 7.2–7.4 (0.8 mM Na_2HPO_4, 1.74 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl), and incubated with biotin-labeled secondary antibodies at 1:100 dilution. Next, the signals were enhanced using the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min, and immune reactions were visualized with the Liquid DAB+ substrate kit (Dako Schweiz AG, Baar, CH). Counter-staining was performed with hematoxylin. Next, dehydration was performed in an ethanol series and xylene, and mounted using Histokit (Assistant, Osterode, Germany). Detailed information about commercially available primary and secondary antibodies, antigen retrieval and concentrations is given in Table 2. All groups within one experiment were simultaneously stained with Liquid DAB+ substrate kit (Dako) for the same target using the same development time.

All slides were qualitatively evaluated for localization of the respective target protein and representative pictures were taken. Two researchers with good experience in canine uterine and placental histology evaluated the signals of target genes independently, using an ordinal system of none (–), weak (+/–), strong (+) and very strong (++) present (Fig. 5).

To identify the specific localization of TIMP2 und TIMP4 in the canine placenta, 4 consecutive slides (2–3 µm thickness) were prepared from placenta in the prepartum luteolysis group. Each consecutive slide was stained with a different primary antibody (TIMP2, TIMP4, vimentin (VIM), pan-cytokeratin (pan-CYT)). Vimentin was used as a marker of mesenchymal cells in order to differentiate between different placental cellular compartments. It stains positively for decidual cells, endothelial cells and fibroblasts. Cytokeratin served as a marker for epithelial cells, staining positively in fetal trophoblast. The staining and evaluation procedures were as described earlier.

Results

mRNA expression of ECM proteins in canine uterus, inter-Pl and Ut-Pl compartments at selected time points during pregnancy and prepartum luteolysis

In all samples, the mRNA expression of ECM proteins, cell adhesion molecules and modulators of ECM was detectable by applying semi-quantitative (TaqMan) PCR.

To evaluate embryo-induced changes in the uterus, a pairwise comparison between non-pregnant and early pregnant (pre-attachment) bitches was performed. In this contrast, *FN1* was significantly suppressed ($P=0.01$) by the presence of embryos during early pregnancy (pre-attachment), in contrast to increased *ECM1* and *TIMP4* expression ($P=0.01$) (Fig. 1). *COL1*, *COL3*, *COL4*, *Cx26* and *-43* were not affected at the beginning of early pregnancy ($P>0.05$, not shown). Effects of the presence of embryos on *LAMA2* and *TIMP2* mRNA expression in the pre-implantation uterus were investigated previously (Graubner *et al.* 2017*a*) and were, therefore, excluded from the present study.

Table 2 List of primary and secondary antibodies used for indirect immunohistochemistry (IHC).

Antibody	Company	Reference number	Antigen retrieval	Dilution
Collagen I (COL1)	Rockland	31680	PIER 60 min	1:800
Collagen III (COL3)	Quartett	292302	PIER 45 min	1:200
Collagen IV (COL4)	Quartett	250424	PIER 60 min	1:500
Laminin 2 alpha (LAMA2)	Bioss Antibodies	bs-8561R	PIER 90 min	1:300
Fibronectin 1 (FN1)	Novus Biologicals	NBP1-91258	PIER 30 min	1:300
alpha Smooth muscle actin (aSMA)	Dako	M0851	HIER	1:100
Connexin 26 (Cx26)	Thermo Fisher	QC215178	PIER 5 min	1:200
Connexin 43 (Cx43)	Abcam	AB11370	PIER 15 min	1:750
Tissue inhibitor of matrix metalloproteinase-2 (TIMP2)	Merck Millipore	AB2965	HIER	1:1500
Tissue inhibitor of matrix metalloproteinase-4 (TIMP4)	LifeSpan Biosciences	LS-C116809	HIER	1:750
Anti-vimentin clone vim3B4 (VIM)	Dako	M7020	HIER	1:100
Anti-cytokeratin (pan-CYT)	Dako	Z0622	HIER	1:700
Extracellular matrix protein 1 (ECM1)	Proteintech	11521-1-AP	HIER	1:200
Biotinylated goat anti-rabbit IgG (H+L)	Vector Laboratories	BA-1000	–	1:100
Biotinylated horse anti-mouse IgG (H+L)	Vector Laboratories	BA-2000	–	1:100

HIER, heat-initiated epitope retrieval; PIER, protease-initiated epitope retrieval.

Next, the uterine pre-implantation stage was compared to inter-Pl sites and to the Ut-Pl compartments from different stages of pregnancy (i.e., post-implantation, mid-gestation and prepartum luteolysis). In inter-Pl sites, uterine gene expression of *ECM1* was significantly upregulated at mid-gestation compared with pre- and post-implantation ($P<0.001$); it did not differ at earlier stages of pregnancy ($P>0.05$), i.e., pre- and post-implantation (Fig. 2). The Ut-Pl compartments showed increased expression of the *ECM1* gene in the prepartum luteolysis group compared with pre-implantation uterus and Ut-Pl sites at early post-implantation stage ($P<0.001$ and $P<0.01$, respectively (Fig. 2)).

The uterine expression of all three major collagens, *COL1*, *COL3* and *COL4*, showed highest mRNA abundance at mid-gestation compared with either pre-implantation ($P<0.05$ for *COL1*, $P<0.01$ for *COL3* and $P<0.001$ for *COL4*) or post-implantation ($P<0.05$ for *COL1* and *COL4*). Whereas *COL1* and *COL4* did not differ between pre-implantation and post-implantation, *COL3* was higher in the early post-implantation group ($P<0.05$) (Fig. 2). As for Ut-Pl compartments, early implantation was associated with suppression of *COL1* and *COL3* levels ($P<0.001$ for *COL1* and $P<0.05$ for *COL3*). Whereas they did not differ from post-implantation to mid-gestation, further suppression of both *COL*s mRNA expression was observed at prepartum luteolysis ($P<0.001$ and $P<0.01$ when compared with mid-gestation for *COL1* and *COL3*, respectively) (Fig. 2). In contrast, the expression of *COL4* in the Ut-Pl comparison was unaffected at all examined stages of pregnancy ($P>0.05$).

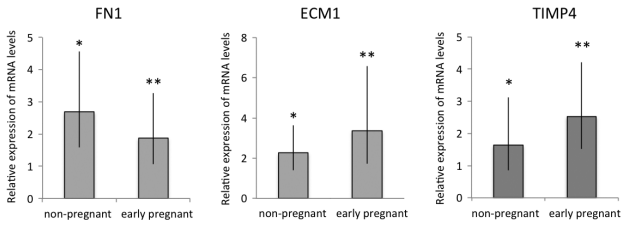


Figure 1 Expression of selected extracellular matrix (ECM) genes in the canine pre-implantation uterus (days 10–12 of pregnancy) affected by the presence of free-floating embryos, compared with gene expression in their non-pregnant counterparts as determined by Real-Time (TaqMan) PCR. *FN1*, fibronectin 1; *ECM1*, extracellular matrix protein 1; *TIMP4*, tissue inhibitor of metalloproteinases 4. An unpaired, two-tailed Student's *t*-test was applied. $P<0.05$ was defined as significant. Bars with different asterisks differ at $P=0.01$. Numerical data are presented as geometric means Xg ± geometric standard deviation (S.D.).

FN1 showed similar distribution patterns at inter-Pl sites as *COL1*, *COL3* and *COL4*. Following a more or less stable expression at inter-Pl at early gestation (i.e., during pre-implantation and post-implantation), its uterine mRNA levels were highest at mid-gestation compared with previous gestational stages ($P<0.05$ and $P<0.001$ for pre-implantation and post-implantation, respectively) (Fig. 3). At placentation sites (Ut-Pl compartments), the prepartum luteolysis was associated with downregulation of *FN1* compared with mid-gestation ($P<0.001$) (Fig. 3).

The uterine mRNA levels of *LAMA2* were not significantly changed ($P>0.05$) during pre-implantation and post-implantation at the inter-Pl sites, but decreased thereafter, from post-implantation to mid-gestation ($P<0.001$) (Fig. 3). *LAMA2* was strongly modulated at implantation sites, decreasing at Ut-Pl following early implantation ($P<0.001$), but it was not further changed toward prepartum luteolysis (Fig. 3).

The uterine levels of *Cx26* were lowest at pre-implantation. They increased, however, significantly ($P<0.001$) at inter-Pl sites, showing the highest mRNA abundance post implantation (Fig. 3). Although apparently decreased at mid-gestation, *Cx26* levels were still more highly represented at mid-gestation compared to pre-implantation ($P<0.001$). When compared with Ut-Pl, the expression of *Cx26* was also significantly increased at implantation sites ($P<0.001$) and maintained

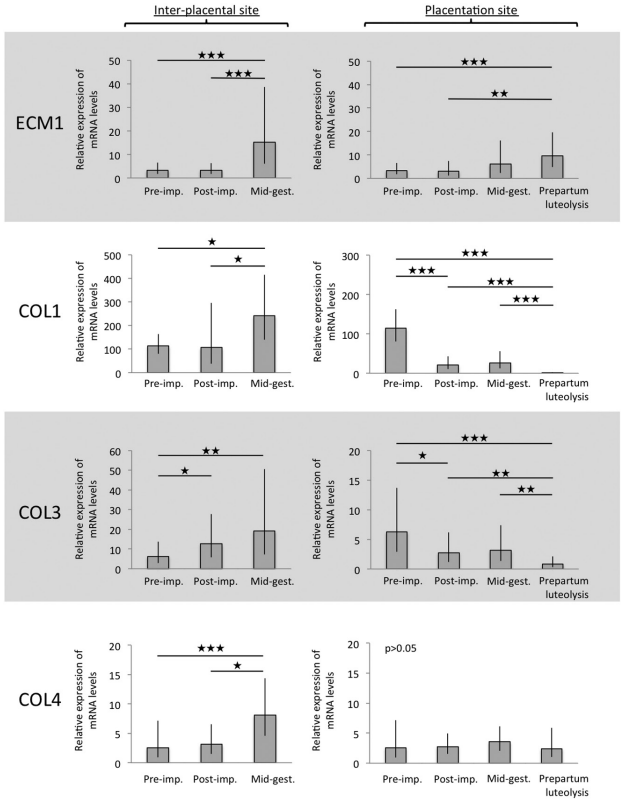


Figure 2 Expression of extracellular matrix protein 1 (*ECM1*), collagen (*COL*) *1*, *-3* and *-4* as determined by Real-Time (TaqMan) PCR in the canine uterus during different times of pregnancy. Two comparisons are presented for each gene: First, the pre-implantation (pre-imp.) stage was compared to the inter-placental sites during post-implantation (post-imp.) and mid-gestation (mid-gest.), and next to the utero-placental compartments (placentation sites) of post-implantation (post-imp.), mid-gestation (mid-gest.) and prepartum luteolysis. Asterisks indicate ⋆$P<0.05$, ⋆⋆$P<0.01$, ⋆⋆⋆$P<0.001$. Numerical data are presented as geometric means Xg ± geometric standard deviation (S.D.).

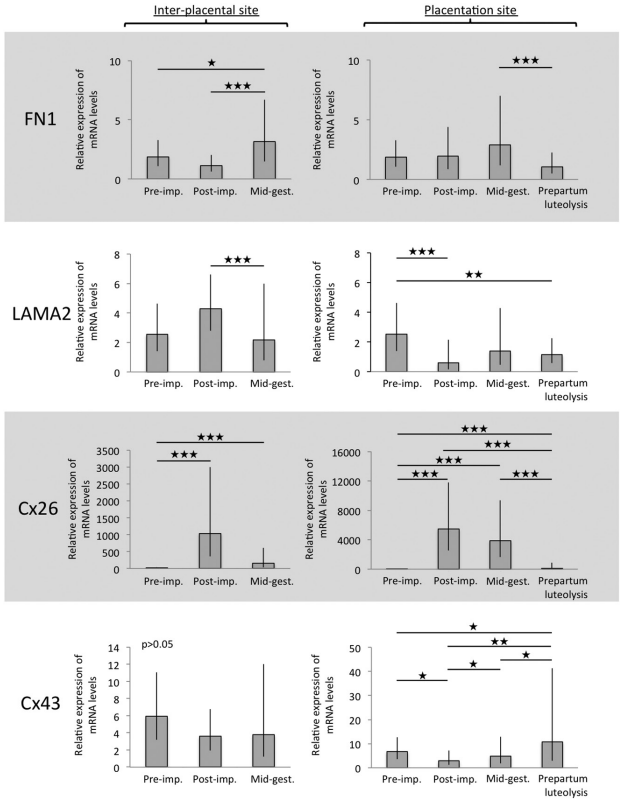


Figure 3 Expression of fibronectin 1 (*FN1*), laminin alpha 2 (*LAMA2*), connexin (*Cx*) *26* and *-43* as determined by Real-Time (TaqMan) PCR in canine uterus during different times of pregnancy. Two comparisons are presented for each gene: first, the pre-implantation (pre-imp.) stage was compared to inter-placental sites during post-implantation (post-imp.) and mid-gestation (mid-gest.), and next to the utero-placental compartments (placentation sites) of post-implantation (post-imp.), mid-gestation (mid-gest.) and prepartum luteolysis. Asterisks differ at ⋆$P<0.05$, ⋆$P<0.01$, ⋆⋆⋆$P<0.001$. Numerical data are presented as geometric means Xg ± geometric standard deviation (S.D.).

its high mRNA levels until mid-gestation. The decrease toward prepartum luteolysis was significant compared with Ut-Pl during post-implantation and mid-gestation ($P<0.001$ and $P<0.001$, respectively) (Fig. 3).

Uterine expression of the *Cx43* gene did not differ significantly at inter-Pl sites ($P>0.05$) (Fig. 3). It was, however, significantly modulated in Ut-Pl compartments. Thus, implantation was associated with suppression of *Cx43* expression at placentation sites compared with the pre-implantation uterus ($P<0.05$). This expression continued, however, to increase at Ut-Pl gradually toward prepartum luteolysis, showing significant differences from post-implantation toward mid-gestation ($P<0.05$) and from mid-gestation to prepartum luteolysis ($P<0.05$).

As for other genes, i.e., *FN1*, *COL1*, *-3*, *-4*, the uterine mRNA encoding for *TIMP2* was highest at mid-gestation ($P<0.01$ and $P<0.001$ compared with pre-implantation and post-implantation, respectively (Fig. 4). *TIMP4* mRNA was already strongly induced at inter-Pl after implantation ($P<0.001$) and maintained its high levels through mid-gestation (Fig. 4). At placentation sites, both *TIMP2* and *TIMP4* were strongly modulated and showed a gradual increase with progression of pregnancy, starting with early gestational stages until prepartum luteolysis when it was highest ($P<0.01$ and $P<0.05$ when compared with post-implantation) (Fig. 4).

Localization of ECM proteins in the canine uterus and placenta

Localization and distribution patterns of COL1, COL3, COL4, FN1, ECM1, LAMA2, Cx26, Cx43, TIMP2 and TIMP4 were investigated at the protein level by applying IHC. All experimental groups were examined. A summary of the findings is presented descriptively in Fig. 5.

COL1 and COL3 were detectable in all maternal and fetal stromal compartments and in myometrium (Fig. 6 and Supplementary Figure 1, see section on supplementary data given at the end of this article, respectively). While at all compared stages of pregnancy (including non-pregnant controls at days 10–12), COL1 staining appeared to be evenly distributed, the intensity of COL3 was generally weaker compared with COL1.

COL4 was clearly detectable throughout gestation and was targeted to the basal lamina of blood vessels (Supplementary Figure 2D and G) in all compartments. Additionally, COL4 was detected in the myometrium of all groups with staining intensity varying, however, individually (Supplementary Figure 2B, F and I).

ECM1 was weakly detectable in luminal epithelium in the non-pregnant group (in one sample it was below the detection limit) (Fig. 7A and B). In early pregnancy, strong signals were detectable in luminal epithelium and in superficial and deep glands (Fig. 7C and D). During mid-gestation and at prepartum luteolysis (Fig. 7E and H), it was only weakly detectable in the glandular epithelium of the superficial glands. At prepartum luteolysis, ECM1 was abundantly present in the connective tissue layer separating superficial glands from deep glands (Fig. 7H). Following implantation (shown at mid-gestation) (Fig. 7F) and at prepartum luteolysis (Fig. 7I), signals were detectable in glandular epithelium of deep glands. Placental localization of ECM1 was in cytotrophoblast (shown at mid-gestation and prepartum luteolysis) (Fig. 7G and J).

Uterine endometrial expression of Cx43 was localized predominantly in epithelial and myometrial compartments (Fig. 8). The luminal epithelium (Fig. 8A and C) and the epithelial cells of superficial and deep glands (Fig. 8A, B, C, D, E, F, H and I) stained strongly for Cx43. Additionally, Cx43 was present in the *tunica intima* of maternal blood vessels in all samples. In placenta (Fig. 8G and J), Cx43 was diffusely present in cytotrophoblast and syncytiotrophoblast and decidual cells. The strongest placental signals were found in the *tunica intima* of blood vessels (Fig. 8G and J).

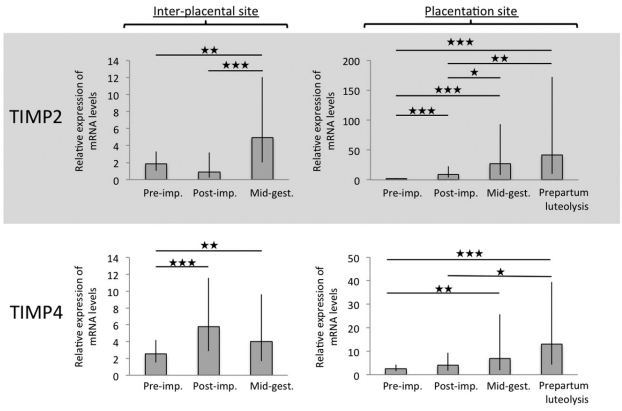


Figure 4 Expression of tissue inhibitor of metalloproteinase (*TIMP*) *2* and *-4* as determined by Real-Time (TaqMan) PCR in canine uterus during different times of gestation. Two comparisons are presented for each gene: First, the pre-implantation (pre-imp.) stage was compared to the inter-placental sites during post-implantation (post-imp.) and mid-gestation (mid-gest.), and next to the utero-placental compartments (placentation sites) of post-implantation (post-imp.), mid-gestation (mid-gest.) and prepartum luteolysis. Asterisks differ at ⋆$P<0.05$, ⋆⋆$P<0.01$, ⋆⋆⋆$P<0.001$. Numerical data are presented as geometric means Xg ± geometric standard deviation (S.D.).

Regarding TIMP2, it was only weakly expressed in the non-pregnant uterus (sometimes below detection limits) (Fig. 9A and B). In contrast, in the early pregnant uterus, at days 10–12 (prior to attachment), its expression was clearly detectable in the luminal epithelium, and superficial and deep glands (Fig. 9C and D). Myometrial signals were weak in both groups (Fig. 9B and D). Following implantation (shown at mid-gestation), endometrial TIMP2 was detectable in epithelial compartments of superficial and deep uterine glands (Fig. 9E and F); stromal compartments of the connective tissue layer appeared only weakly stained (Fig. 9E). At prepartum luteolysis, uterine TIMP2 signals were weaker in the epithelium of superficial glands (Fig. 9H). However, strong staining was observed in the connective tissue layer separating the superficial from deep glands (Fig. 9H). Myometrial signals were weak (Fig. 9F and I). Placental TIMP2 at mid-gestation was distributed ubiquitously and was found in cytotrophoblast and syncytiotrophoblast, in decidual cells and in vascular endothelial cells (*tunica intima*) (Fig. 9G). During prepartum luteolysis, placental expression was predominantly found in endothelial cells of maternal vessels (Fig. 9J). Sporadic nuclear staining was detected in the nuclei of myocytes, which did not allow further interpretation.

Compared with TIMP2, TIMP4 appeared to stain generally more weakly in all experimental groups and tissue compartments. Although weakly represented, uterine expression was localized in the luminal epithelium (Fig. 10A and C). Somewhat stronger staining was found in superficial and deep glands (Fig. 10A, B, C, D and F). Stronger TIMP4 signals were found during prepartum luteolysis in the connective tissue layer separating superficial from deep glands (Fig. 10H). It also appeared to be induced in deep uterine glands (Fig. 10I).

Within Ut-Pl compartments, placental TIMP4 was found in cytotrophoblast (shown at mid-gestation and during prepartum luteolysis; Fig. 10G and J). For Supplementary Figure 3, consecutive cross-sections of Ut-Pl compartment were prepared during prepartum luteolysis and were stained with either vimentin (VIM; Supplementary Figure 3C), pan-cytokeratin (pan-CYT; Supplementary Figure 3D), TIMP2 or TIMP4, allowing better differentiation between cellular tissue compartments stained by particular antibodies. In particular, localization patterns of both

	COL1	COL3	COL4	FN1	ECM1	LAMA2	Cx26	Cx43	TIMP2	TIMP4	aSMA
NON-PREGNANT ***ENDOMETRIUM***											
Luminal epithelium	-	-	-	+	+	+	+/-	+	+/-	+/-	-
Stroma	+	+	-	+	-	+	-	-	-	-	+
Glandular epithelium	-	-	-	+	-	+	+/-	+	+/-	+	-
Blood vessels	-	-	+	-	-	+	-	+	+	-	++
MYOMETRIUM											
Smooth muscle cells	-	-	-	-	-	-	+	+	+/-	-	++
Stroma	+	+	+	+	-	+	-	-	+	-	-
Blood vessels	-	-	+	-	-	+	-	+	+	-	++
PRE-ATTACHMENT ***ENDOMETRIUM***											
Luminal epithelium	-	-	-	+	++	+	+	+	++	+/-	-
Stroma	+	+	-	+	-	+	-	-	-	-	-
Glandular epithelium	-	-	-	+	+	+	+	+	++	+	-
Blood vessels	-	-	+	-	-	+	-	+	+	-	+
MYOMETRIUM											
Smooth muscle cells	-	-	-	-	-	+	+	+	+	-	+
Stroma	+	+	+/-	+	-	+	-	-	+	-	-
Blood vessels	-	-	+	-	-	+	-	+	+	-	++
MID-GESTATION ***PLACENTA***											
Cytotrophoblast cells	-	-	-	+	+	-	+	+/-	+	+	-
Syncytiotrophoblast	-	-	-	+	-	-	+	+/-	+	-	-
Decidual cells	-	-	-	+	-	-	+	+/-	+	-	++
Blood vessels	-	-	+	+	-	+	+	++	+	-	+
Stroma	+	+	-	+	-	+	-	-	-	-	-
ENDOMETRIUM											
Epithelium of superficial glands	-	-	-	+	+	+	++	+	+	+/-	-
Connective tissue layer separating superficial from deep glands	+	+	-	+	-	++	-	+	+/-	-	-
Epithelium of deep glands	-	-	-	+	+	+	+	+	+	+	-
Blood vessels	-	-	+	+	-	+	+/-	++	+	-	+
MYOMETRIUM											
Smooth muscel cells	-	-	-	-	-	+	+	+	+	-	+
Blood vessels	-	-	+	-	-	+	-	+	+	-	++
Stroma	+	+	+	+	-	+	-	-	+	+	-
PREPARTUM LUTEOLYSIS ***PLACENTA***											
Cytotrophoblast cells	-	-	-	+	+	-	++	+	+	+	-
Syncytiotrophoblast	-	-	-	+	-	-	+	+	+	-	-
Decidual cells	-	-	-	+	-	-	+	+	+	-	++
Blood vessels	-	-	+	+	-	+	+/-	++	++	-	++
Stroma	+	+	-	+	-	+	-	-	-	-	-
ENDOMETRIUM											
Epithelium of superficial glands	-	-	-	+	+	+	++	+	+/-	+/-	-
Connective tissue layer separating superficial from deep glands	+	+	-	+	++	+	+	+	++	++	-
Epithelium of deep glands	-	-	-	+	+	+	+	+	+	++	-
Blood vessels	-	-	+	-	-	+	+	++	+	-	++
MYOMETRIUM											
Smooth muscel cells	-	-	-	-	-	+	+	+	+	-	+
Blood vessels	-	-	+	-	-	+	+	+	+	-	+
Stroma	+	+	+/-	+	-	+	-	-	+	+	-

Figure 5 Tabular, descriptive presentation of localization patterns of extracellular matrix proteins (ECM) as determined by immunohistochemistry (IHC) in canine uterine and placental compartments. (–), not present; (+/–), weakly present; (+), strongly present; (++), very strongly present; aSMA, alpha-smooth muscle actin; COL, collagen; Cx, connexin; ECM1, extracellular matrix protein 1; FN1, fibronectin 1; LAMA2, laminin alpha 2; TIMP, tissue inhibitor of matrix metalloproteinase.

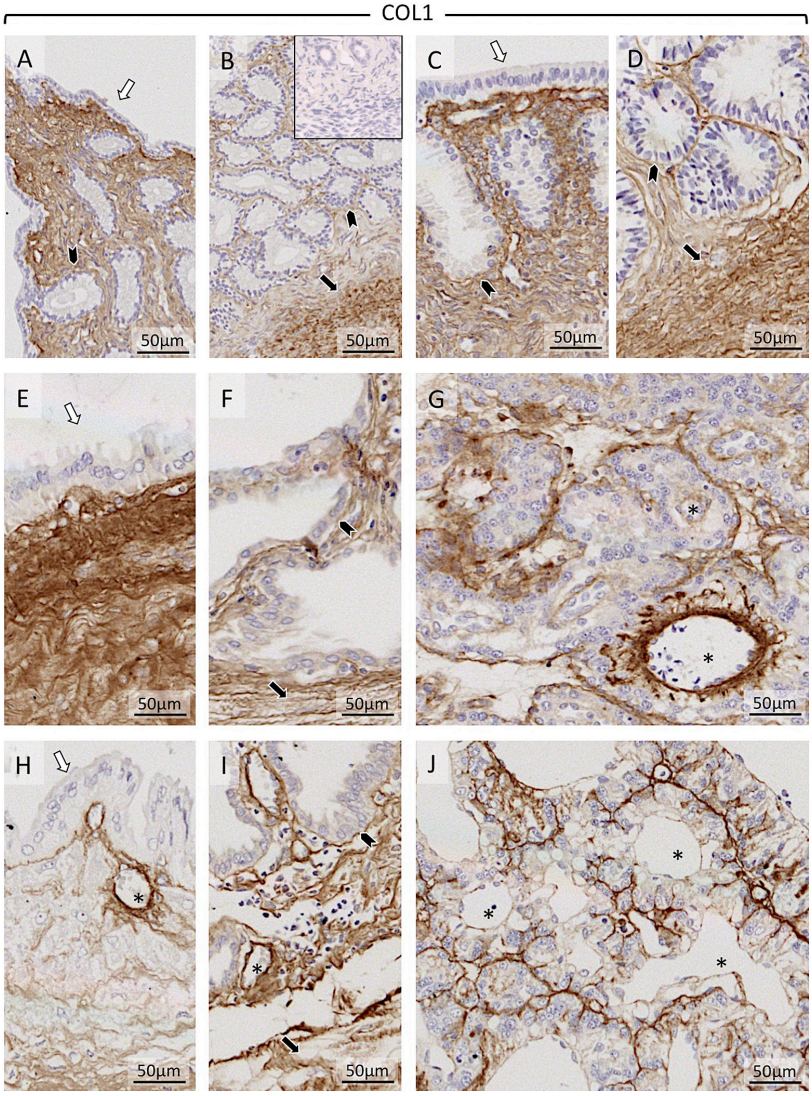


Figure 6 Representative pictures of immunohistochemical detection of collagen 1 (COL1) in the canine uterus and utero-placental compartments (Ut-Pl) at selected time points during pregnancy: the non-pregnant canine uterus at early diestrus (A and B), early pregnant (pre-implantation) canine uterus (C and D), Ut-Pl units during mid-gestation (E, F and G), and in Ut-Pl compartments at prepartum luteolysis (H, I and J); (A and C) luminal part of uterus with surface area; (B, D, F and I) deep glands area at the border with the myometrium; (E and H) the connective tissue layer and part of the glandular area above it (superficial glands, so-called glandular chambers); (G and J) placental compartments. Non-pregnant (A and B), and before implantation and placentation (C and D): open arrows = surface (luminal) epithelium; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium. After implantation and placentation (E, F, G, H, I and J): open arrows = glandular epithelium of superficial glands; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium of deep glands; asterisk = blood vessel; Collagen 1 was clearly detectable throughout pregnancy in all stromal compartments. Before implantation and placentation, strong signals were detected in stroma and myometrium of both non-pregnant (A and B) and pregnant dogs (C and D). Following implantation and placentation, strong signals were localized in the connective tissue layer (stromal area) separating superficial from deep glands (E and H) as well as in myometrial (F and I) and placental stromal (G and J) compartments. Inset in picture B shows a negative (isotype) control.

factors within canine placenta are clearly presented, with TIMP2 targeting to vascular endothelial cells and cytotrophoblast, presenting only weak signals in syncytiotrophoblast and maternal decidual cells (Supplementary Figure 3A). Clear TIMP4 signals were localized in cytotrophoblast (Supplementary Figure 3B).

In all samples, strong signals for aSMA were found in myometrium and the media of blood vessels (Supplementary Figure 4). Additionally, in the placenta aSMA was present in decidual cells, as shown at mid-gestation and prepartum luteolysis (Supplementary Figure 4G and J).

FN1 was detectable throughout pregnancy. In general, stronger signals were found in epithelial than stromal compartments. The luminal epithelium of the non-pregnant group (Supplementary Figure 5A) was more strongly stained compared with the pre-attachment group (Supplementary Figure 5C). The myometrium stained weakly in all experimental groups and was below detection limits in some samples (Supplementary Figure 5B, F and I). Additionally, diffuse signals for FN1 were observed throughout all cellular placental compartments, i.e., syncytio- and cytotrophoblast, decidual cells and blood vessels (Supplementary Figure 5G and J).

LAMA2 (Supplementary Figure 6) was clearly present in all stromal compartments and in the media of blood vessels throughout pregnancy. Additionally, it was detectable in glandular epithelial cells in all groups. In the placental part of Ut-Pl compartments, LAMA2 staining was stronger in the stroma than in blood vessels (Supplementary Figure 6G and J).

Regarding Cx26, this protein was present in epithelial compartments throughout pregnancy (Supplementary

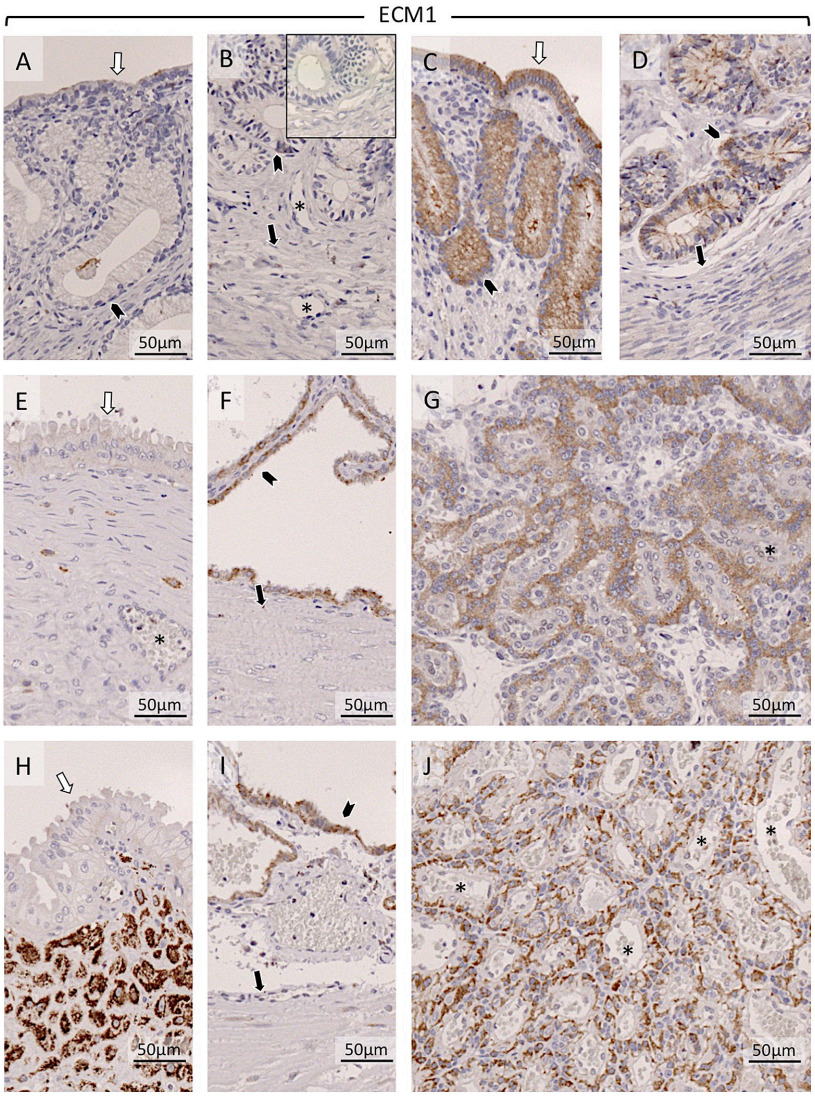


Figure 7 Representative pictures of immunohistochemical detection of extracellular matrix protein 1 (ECM1) in the canine uterus and utero-placental compartments (Ut-Pl) at selected time points during pregnancy: in the non-pregnant canine uterus at early diestrus (A and B), early pregnant (pre-implantation) canine uterus (C and D), Ut-Pl units during mid-gestation (E, F and G), and in Ut-Pl compartments at prepartum luteolysis (H, I and J); (A and C) luminal part of uterus with uterine surface area; (B, D, F and I) deep glands area at the border with the myometrium; (E and H) the connective tissue layer and part of the glandular area above it (superficial glands, so-called glandular chambers); (G and J) placental compartments. Non-pregnant (A and B), and before implantation and placentation (C andD): open arrows = surface (luminal) epithelium; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium; asterisk = blood vessel. After implantation and placentation (E, F, G, H, I and J): open arrows = glandular epithelium of superficial glands; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium of deep glands; asterisk = blood vessel; ECM1 was weakly detectable in luminal epithelium in the non-pregnant group (A and B). In early pregnancy, strong signals were detectable in luminal epithelium and in superficial and deep glands (C and D). During mid-gestation (E) and at prepartum luteolysis (H), ECM1 was weakly detectable in glandular epithelium of the superficial glands. At prepartum luteolysis, ECM was strongly present in the connective tissue layer separating superficial glands from deep glands (H). In mid-gestation (F) and at prepartum luteolysis (I), ECM1 was detectable in the glandular epithelium of deep glands. Placental localization of ECM1 was in cytotrophoblast, shown at mid-gestation (G) and prepartum luteolysis (J). Inset in picture B shows respective negative (isotype) control.

Figure 7). Generally, signals were weaker in stromal and myometrial compartments. Cx26 was also detectable in endometrial epithelial cells. Following implantation, as shown during mid-gestation and at prepartum luteolysis, this protein was strongly locally induced in the epithelial cells of glandular chambers immediately above the connective tissue layer (Supplementary Figure 7E and H). Placental Cx26 was present diffusely in cytotrophoblast and syncytiotrophoblast and in decidual cells (indicated in Supplementary Figure 6J).

Discussion

The effects of modifications of uterine ECM on canine pregnancy have not been considered for many years. Because different tissues consist of dissimilar mixed combinations of cells, which evolutionarily developed from different pedigrees, the configuration of ECM varies among tissues (Abedin & King 2010). These ECM components are involved in the regulation of cell growth and differentiation, act as extracellular storage of hormones and are involved in the activation of signaling cascades (reviewed in Hubmacher & Apte 2013, da Anunciacao *et al.* 2017). In the uterus, the major ECM components are collagens, proteoglycans, hyaluronan and glycoproteins (Leoni *et al.* 1990, Oliveira *et al.* 2015, Franczyk *et al.* 2017). It is worth noting that in mammals, the uterine ECM composition changes dynamically depending on the reproductive status (i.e., non-pregnant vs pregnant) (Cabrol *et al.* 1985, Leoni *et al.* 1990, Hjelm *et al.* 2002, Boos *et al.* 2003), and in some species, such as humans and cattle,

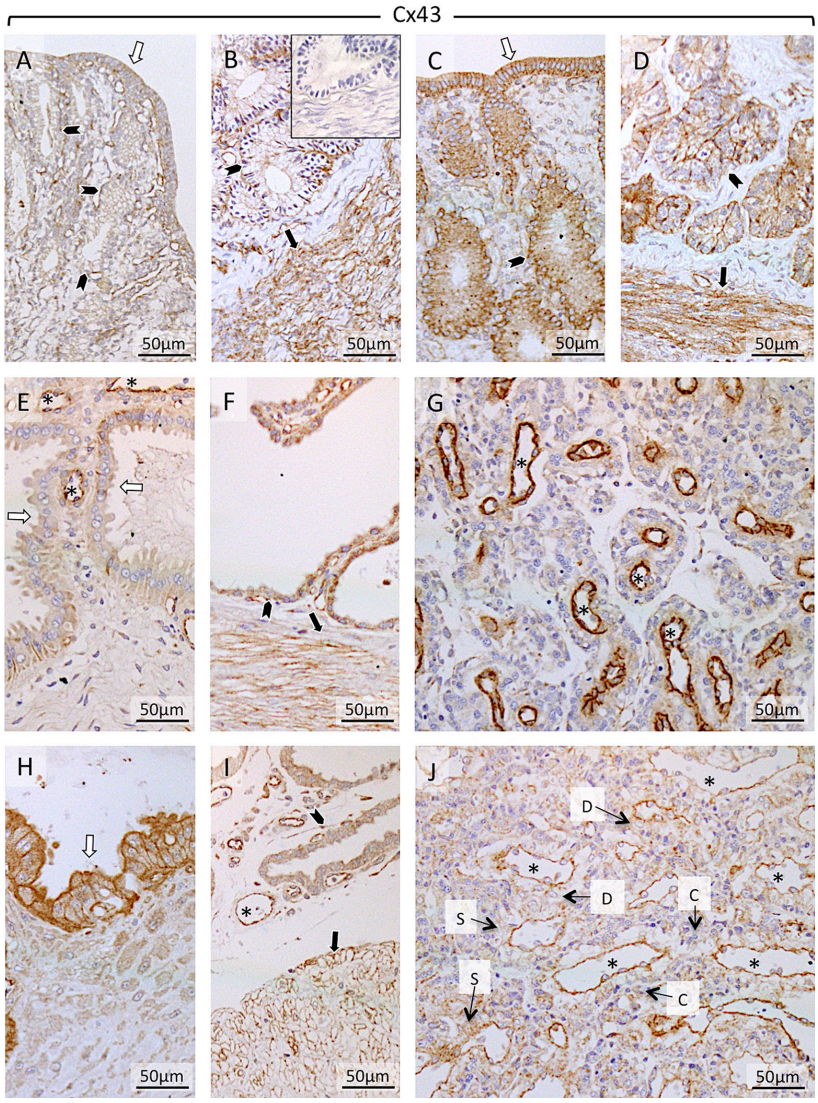


Figure 8 Representative pictures of immunohistochemical detection of cell adhesion molecule connexin 43 (Cx43) in the canine uterus and utero-placental compartments (Ut-Pl) at selected time points during pregnancy: in the non-pregnant canine uterus at early diestrus (A and B), early pregnant (pre-implantation) canine uterus (C and D), Ut-Pl units during mid-gestation (E, F and G), and in Ut-Pl compartments at prepartum luteolysis (H, I and J); (A and C) luminal part of uterus with uterine surface area; (B, D, F and I): deep glands area at the border with the myometrium; (E and H) the connective tissue layer and part of the glandular area above it (superficial glands, so-called glandular chambers); (G and J) placental compartments. Non-pregnant (A and B), and before implantation and placentation (C and D): open arrows = surface (luminal) epithelium; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium. After implantation and placentation (E, F, G, H, I and J): open arrows = glandular epithelium of superficial glands; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium of deep glands; asterisk = blood vessel; D = decidual cell; S = syncytiotrophoblast; C = cytotrophoblast; Cx43 was present in epithelial compartments and myometrial compartments. The luminal epithelium (A and C) and the epithelial cells of superficial and deep glands (A, B, C, D, E, F, H and I) stained strongly for Cx43. Additionally, the protein was present in tunica intima of blood vessels in all samples. Placental Cx43 localization was diffusely detected in cyto- and syncytiotophoblast and decidual cells (G and J), additionally strong signals for Cx43 were detected in the intima and media of placental blood vessels. Inset in picture B shows respective negative (isotype) control.

the composition of the uterine ECM is cycle dependent (Boos 2000, Curry & Osteen 2001). In fact, most details about uterine ECM modifications and functions during pregnancy are known only for humans and rodents. In these species, in early pregnancy, the uterine ECM influences trophoblast invasion (Johnson *et al.* 2003) and remodeling of decidua (Damsky *et al.* 1993, Lala & Nandi 2016, Smith *et al.* 2016), while the ECM is modulated by matrix metalloproteinases (MMPs) of fetal (trophoblast) origin. As for the canine non-pregnant and pregnant uterus and placenta, the time- and organ-specific expression of MMP2 and -9 have been investigated (Beceriklisoy *et al.* 2007, Fellows *et al.* 2012, Diessler *et al.* 2017). Their expression patterns indicate pregnancy-associated modifications of ECM.

The present study explored the spatiotemporal expression of ECM components selected from our preceding microarray analysis (Graubner *et al.* 2017*a*), and known for their involvement in modulating uterine and placental functions in other animals, in the canine uterus and placenta throughout pregnancy. Quantitative assessment was done by qPCR, and cellular localization was investigated qualitatively by IHC. The antibodies applied herein proved unsuitable for Western blot analysis. It appears plausible that linearization of proteins by reducing agents during Western blot preparation affects the structure of target epitopes, thereby preventing antibody binding.

Structural and adhesive proteins of the ECM

Expression of the following proteins was assessed: COL1, COL3, COL4, ECM1, FN1, LAMA2 and aSMA. Free-floating embryos were previously reported to increase the uterine expression of *LAMA2* (Graubner *et al.* 2017*a*). Here, the uterine adjustments during early

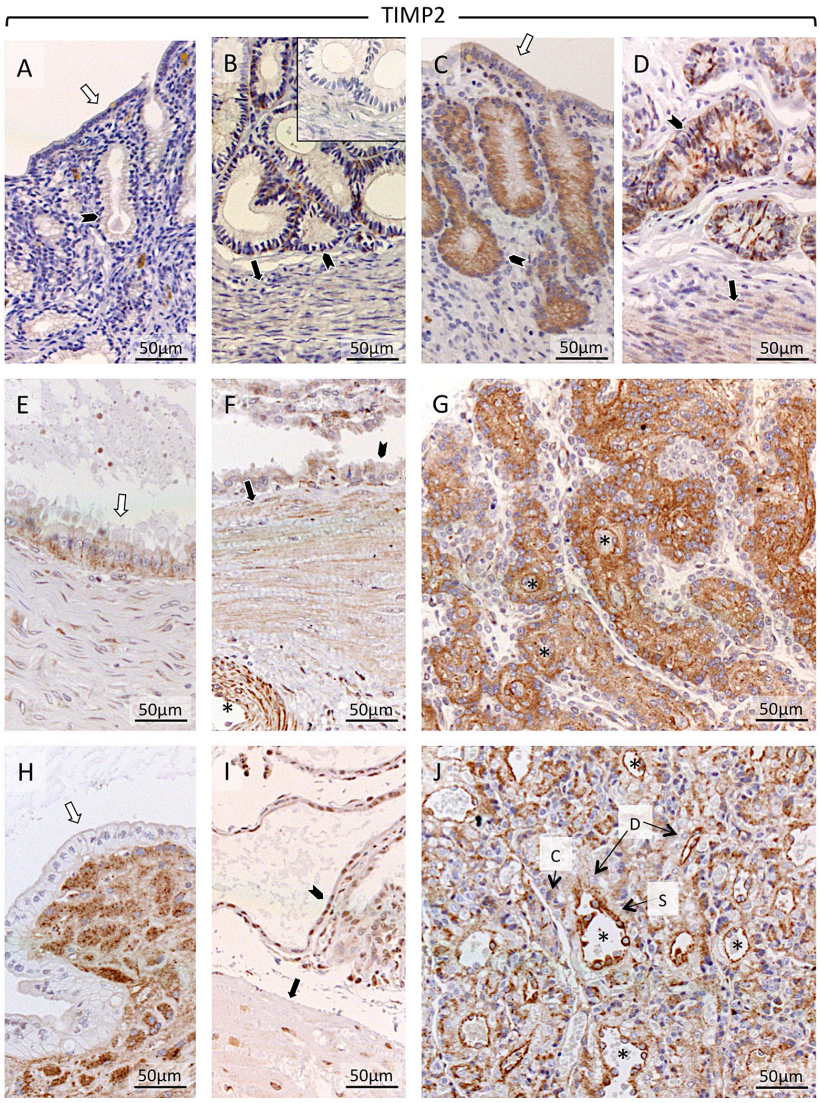


Figure 9 Representative pictures of immunohistochemical detection of tissue inhibitor of metalloproteinases 2 (TIMP2) in the canine uterus and utero-placental compartments (Ut-Pl) at selected time points during pregnancy: in the non-pregnant canine uterus at early diestrus (A and B), early pregnant (pre-implantation) canine uterus (C and D), Ut-Pl units during mid-gestation (E, F and G), and in Ut-Pl compartments at prepartum luteolysis (H, I and J); (A and C) luminal part of uterus with uterine surface area; (B, D, F and I) deep glands area at the border with the myometrium; (E and H) the connective tissue layer and part of the glandular area above it (superficial glands, so-called glandular chambers); (G and J) placental compartments. Non-pregnant (A and B), and before implantation and placentation (C and D): open arrows = surface (luminal) epithelium; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium. After implantation and placentation (E, F, G, H, I and J): open arrows = glandular epithelium of superficial glands; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium of deep glands; asterisk = blood vessel; D = decidual cell; S = syncytiotrophoblast; C = cytotrophoblast; In the non-pregnant group, TIMP2 was weakly detectable in the deep glands (B). In early pregnancy, TIMP2 was clearly detectable in luminal epithelium and epithelium of superficial (C) and deep glands (D), and myometrial compartments (D). At mid-gestation, TIMP2 was detectable in the glandular epithelium of the superficial glands (E), but not in the stroma of the connective tissue layer separating superficial from deep glands (E). At prepartum luteolysis, TIMP2 signals appeared weaker (compared to mid-gestation) in the luminal epithelium of the superficial glands; additionally, strong signals were detected in the connective tissue layer (H). At mid-gestation (F) TIMP2 appeared in the *tunica media* of blood vessels in the myometrium. At mid-gestation (F) and at prepartum luteolysis (I), TIMP2 was present in the myometrium and in deep glands. In placenta at mid-gestation (G) and at prepartum luteolysis (J), TIMP2 was present in cyto- and syncytiotrophoblast, decidual cells and the intima of blood vessels. In placenta the endothelium appeared to stain strongest for TIMP2. Inset in picture B shows the respective negative (isotype) control.

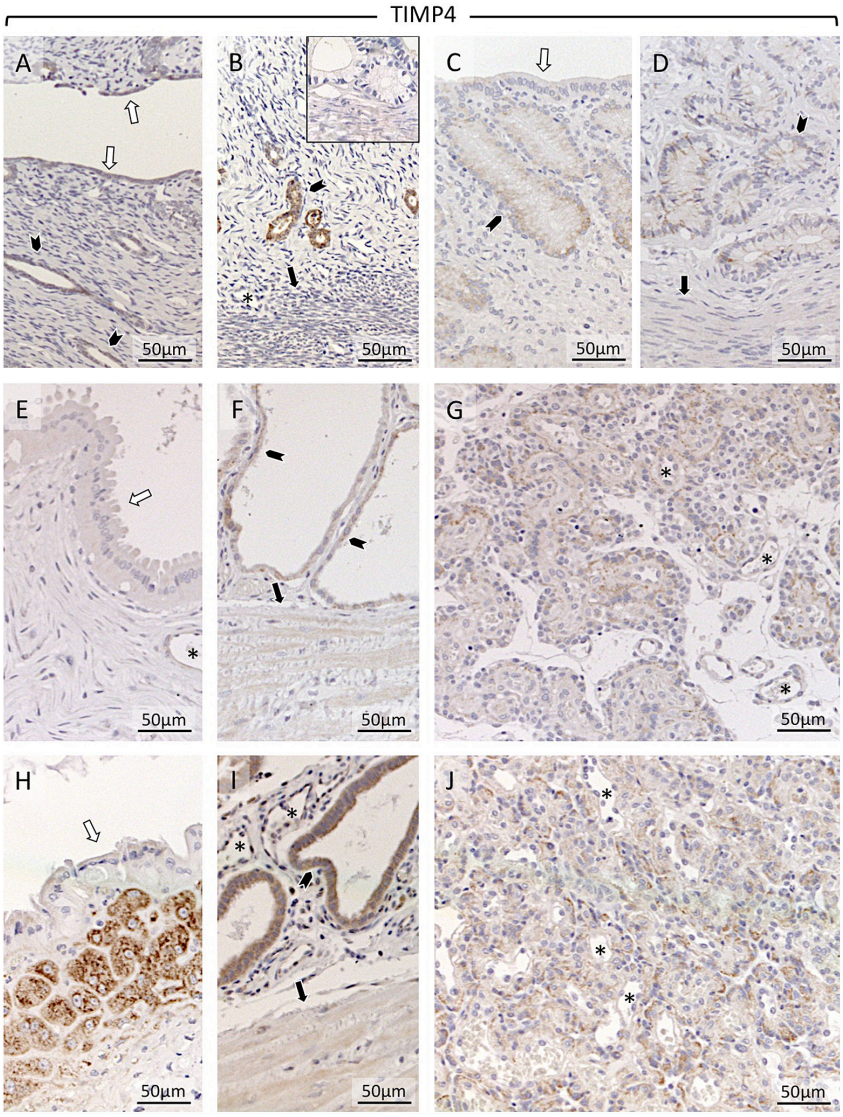


Figure 10 Representative pictures of immunohistochemical detection of tissue inhibitor of metalloproteinases 4 (TIMP4) in the canine uterus and utero-placental compartments (Ut-Pl) at selected time points during pregnancy: in the non-pregnant canine uterus at early diestrus (A, B), early pregnant (pre-implantation) canine uterus (C and D), Ut-Pl units during mid-gestation (E, F and G), and in Ut-Pl compartments at prepartum luteolysis (H, I and J); (A and C) luminal surface area; (B, D, F and I) deep glands area at the border of the myometrium; (E and H) the connective tissue layer and part of the glandular area above it (superficial glands, so-called glandular chambers); (G and J) placental compartments. Non-pregnant (A and B), and before implantation and placentation (C and D): open arrows = surface (luminal) epithelium; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium; asterisk = blood vessel. After implantation and placentation (E-J): open arrows = glandular epithelium of superficial glands; solid arrows = myometrium (circular layer); solid arrowhead = glandular epithelium of deep glands; asterisk = blood vessel; Before trophoblast attachment (i.e. at early pregnancy), and in non-pregnant controls, TIMP4 appeared weakly present in the surface epithelium (A and C). Also superficial epithelium stained weakly throughout pregnancy. Stronger signals were seen in the epithelium of deep glands, in particular in non-pregnant dogs (B) and at mid-gestation and in the prepartum luteolysis group (F and I). TIMP4 was strongly detected at prepartum luteolysis in the connective tissue layer separating superficial from deep glands (H). In the placenta, shown at mid-gestation and prepartum luteolysis, TIMP4 was present in the cytotrophoblast (G and J). Inset in picture B shows respective negative (isotype) control.

decidualization were additionally characterized by increased expression of *ECM1*, whereas *FN1* was suppressed. None of the major COLs was affected by the presence of embryos.

Showing distinctly different localization patterns and both being affected by presence of embryos, ECM1 and LAMA2 appear to be involved in the establishment of canine pregnancy. As components of the lamina basalis, increased expression of laminins is a marker of ongoing stromal decidualization in humans (Church *et al.* 1997). Similarly in the dog, even if devoid of apparent morphological differentiation of the uterus, pre-implantation embryos stimulated the expression of LAMA2, predominantly in the uterine stroma. The increased ECM1 expression was abundantly localized in endometrial epithelium. In the human endometrium, its expression during implantation is further stimulated by some of the chemokines critically involved in leukocyte migration and trophoblast invasion, e.g., CX3CL1 and CCL14 (Hannan & Salamonsen 2008). Although not yet investigated, similar functional relationships cannot be ruled out for the canine species. Further effects of ECM1 in the canine uterus might be associated with its proliferative activities. Thus, by interacting with the epidermal growth factor (EGF)-related pathways, ECM1 promotes cellular differentiation and proliferation (Lee *et al.* 2014). Within the canine uterus, the localization patterns of EGF receptor (EGFR) were described previously (Sagsoz *et al.* 2014) and reflect the localization of ECM1 presented herein. A possible functional relationship between these two entities is certainly worth attention and should be addressed in the future.

Regarding the embryo-induced suppression of the cell adhesion molecule *FN1*, similar to the dog, its decrease during early pregnancy also occurs in rodents (Zollinger & Smith 2017), a phenomenon that has been linked to

the early decidualization process around the time of implantation (Grinnell *et al.* 1982). Moreover, reduction of FN1 expression seems to be important to prevent excessive trophoblast invasion into maternal tissues (Kaloglu & Onarlioglu 2010). Despite the less invasive type of canine placentation compared to rodents, the *FN1* decrease observed before trophoblast attachment in dogs could also possibly be linked to similar protective functions in order to control trophoblast invasion. A key mechanism in this context seems to be the cooperation of FN1 and integrin molecules in modulating trophoblast proteolytic activities (through/via) the ECM (Sutherland *et al.* 1993, Fazleabas *et al.* 1997). Interestingly, however, the canine uterine expression of several integrins (ITG) (e.g., ITGA2B, ITGB2 and ITGB3) increases in early pregnancy (Bukowska *et al.* 2011), while FN1 expression is diminished by the presence of embryos.

It needs to be emphasized that, in agreement with our previous findings (Graubner *et al.* 2017*a*), and in contrast to other species including humans and rodents, the above described functional changes in the canine uterus driven by the presence of embryos, take place without any morphologically visible alterations of uterine structures. The deep structural remodeling processes of the canine uterus start following implantation and placentation and are associated with increased expression of COLs (*COL1*, *-3*, and *-4*), *ECM1* and *FN1* in the uterine wall (i.e., inter-Pl). The increased expression of structural and basement membrane components, widely localized in stroma and, depending on the type of COL (e.g., COL1 and -3), in myometrium, seems to relate to the uterine growth as a natural phenomenon resulting from the development of conceptuses.

Conversely, at implantation sites, trophoblast invasion and placentation decreased the expression of two major COLs (COL1 and -3) and LAMA2. This is apparently associated with the remodeling processes and placental development, as all COLs, and in particular the most robust COL1, were clearly detectable in utero-placental stromal compartments.

Similar effects were observed in other animals displaying invasive types of placentation, e.g., in rodents, in which implantation was associated with decreased *COL1* expression at invasion sites, implying the need for *COL1* suppression during remodeling events associated with embryo-induced decidualization and subsequent placentation in these animals (Clark *et al.* 1993).

The decrease in *LAMA2* found in our study additionally indicates loss of stability of the basal lamina, and associated with it migration and differentiation of cells (Kleinman *et al.* 1985, Engvall *et al.* 1990, Aumailley 2013). This differentiates the dog from mammals with more invasive forms of placentation such as rodents and primates. Accordingly, around the time of implantation in the murine uterus, laminin is synthesized by decidual cells (Wewer *et al.* 1985) where it influences trophoblast migration (Zhang *et al.* 2000, Korgun *et al.* 2007). In humans also decidual cells produce laminin (Kisalus *et al.* 1987, Aplin *et al.* 1988, Haouzi *et al.* 2011). At later stages of human gestation, several isoforms of laminin and fibronectin are found in the basal lamina of capillaries and in the stroma and trophoblast cells of the villi (Korhonen & Virtanen 2001). As for the dog, LAMA2 seemed to be more strongly represented in the stromal than epithelial compartments, in particular following implantation.

Besides laminin, the decidualization process in humans is marked by increased expression of aSMA (Oliver *et al.* 1999). This protein seems to be strongly required during pregnancy since there is a relationship between abnormal actin polymerization and pregnancy failure in humans (Montazeri *et al.* 2015). In dogs, aSMA is only weakly present in uterine stromal cells of non-pregnant animals and at the pre-implantation stage. However, following placentation aSMA is strongly expressed in maternal decidual cells and can be used as a cellular marker of decidual cells in the canine species. As a matter of fact, aSMA was also strongly expressed in canine decidualized uterine primary stromal cells *in vitro* (Kautz *et al.* 2015).

Following attachment and invasion of the trophoblast in dogs the girdle placenta is developing. Histologically, at the placentation site a connective tissue layer can be found that separates enlarged superficial glands (so-called glandular chambers) from deeper localized parts of the uterus. It functions as a barrier, protecting deep uterine glands and myometrium from proteolytic activity of the trophoblast. When this barrier is breached, an exaggerated invasion of trophoblast may result in SIPS (Al-Bassam *et al.* 1981). Clearly detectable staining of structural collagens (COL1, and -3) and basal lamina components such as LAMA2 was identified throughout pregnancy in the stroma of this connective tissue layer. Their respective mRNA levels were not modulated from post-implantation toward mid-gestation, but the major collagens, COL1 and -3, were strongly suppressed at the time of prepartum luteolysis. This may be seen as an indicator of preparation for parturition and release of the fetal membranes (placentolysis). A similar role of physiological degradation of collagens during preparation for the release of fetal membranes has been discussed for cattle (Attupuram *et al.* 2016). In fact, a retained placenta can be successfully treated by administration of collagenases into the umbilical artery (Eiler & Hopkins 1993, Attupuram *et al.* 2016).

With regards to *FN1*, which was generally more strongly represented in epithelial compartments, its uterine and placental expression followed the expression patterns exhibited by COLs. This leads to the assumption that the main role of *FN1* in the canine uterus is a bridging function between collagens and other ECM components.

The utero-placental *ECM1* expression increased with the progression of pregnancy and was highest at prepartum luteolysis. Within the placenta, ECM1 stained predominantly in cytotrophoblast, which appeared to be the major source of ECM1 in the Ut-Pl compartment. This resembles results observed in humans where the importance of ECM1 during maintenance of pregnancy and fetal development is underlined by its highest expression in the placenta and fetal heart tissue but not in other organs (e.g., brain, lung, liver, kidney, pancreas or skeletal muscles) (Smits *et al.* 1997). In addition to the aforementioned function of ECM1 in promoting proliferation and differentiation of cells during the onset of pregnancy, at prepartum luteolysis the co-localization of ECM1 with TIMP2- and -4 in the tissue layer separating superficial from deep uterine glands strongly attracted our attention. This spatio-temporal detection is specific to prepartum luteolysis, but not at earlier stages of pregnancy. A possible implication for its function arises from *in vitro* studies showing ECM1-mediated reduction of MMP9 proteolytic activity in a human model (Fujimoto *et al.* 2006). Cumulatively, a protective role of ECM1, possibly interacting with a metalloproteinases activity-balancing system, should be considered.

Connexins (Cx26 and -43)

Connexins (Cx) build gap junction proteins, through which two cells can exchange small molecules, electrical charges and second messengers (Bruzzone *et al.* 1996, Kumar & Gilula 1996, Evans & Martin 2002). The uterine expression of *Cx26* and *-43* has been described during the menstrual cycle in humans as well as during pregnancy in humans, rats and sheep (Winterhager *et al.* 1993, Grummer *et al.* 1994, Jahn *et al.* 1995, Johnson *et al.* 2017). Both hormonal- and embryo implantation-mediated effects were observed (Grummer *et al.* 2004). Successful implantation of embryos in rodents requires a complete suppression of both connexins prior to implantation (Grummer *et al.* 1994, 2004). At the time of implantation connexins are re-induced (Grummer *et al.* 1996). The importance of Cx during establishment of pregnancy has been further strengthened by their abnormal expression patterns reported in pathologies such as recurrent pregnancy loss (Laird 2006, Nair *et al.* 2011).

No such information was so far available regarding the canine uterus and placenta, which prompted us to investigate the expression and distribution patterns of Cx26 and -43 throughout pregnancy. The uterine expression of both *Cx* remained unaffected by the presence of free-floating embryos. The respective proteins were detectable, however, with Cx43 appearing more abundantly expressed than Cx26. Whereas being clearly detectable, *Cx43* was more or less constantly present in the uterine wall; its increasing expression in the Ut-Pl compartment followed placental development and reached its highest levels during prepartum luteolysis. It is noteworthy that, although rather ubiquitously expressed, within the placenta Cx43 mostly targeted to the maternal endothelium. This expression pattern differed strongly from that observed for Cx26, which appeared to be in agreement with observations made in other species exhibiting invasive types of placentation. Thus, the uterine and placental expression of *Cx26* was strongly induced following implantation. Its expression was predominantly localized in the endometrial tissues, both at the placentation sites and at inter-Pl. The prepartum luteolysis was associated with a strong decrease of the respective mRNA levels.

Regarding the possible functions of connexins within the placenta, in humans *Cx43* appears indispensable for proper decidualization and uterine angiogenesis (Laws *et al.* 2008). Additionally, it has been shown *in vitro* that *Cx43* is involved in regulating differentiation of cytotrophoblast to syncytiotrophoblast and plays roles in feto-maternal exchange (Cronier *et al.* 2002). Based on the localization pattern of Cx43 described above, in the dog its involvement in uterine and placental angiogenesis also seems likely, since the strongest signals were noticed in the intima and media of placental blood vessels. The involvement of Cx43 during canine decidualization cannot, however, be ruled out and merits further investigations.

Spatio-temporal expression of TIMP2 and TIMP4

When discussing the development and function of the canine endotheliochorial placenta, the mechanisms controlling the invasive behavior of trophoblast need to be considered. In general, the invasive properties of trophoblast are at least in part provided by MMPs (reviewed in Goldman-Wohl & Yagel 2002, Cohen *et al.* 2006). At parturition, MMPs are additionally associated with placental detachment, as shown in humans and cattle (Eiler & Hopkins 1992, Strauss 2013, Menon *et al.* 2016). In dogs, MMPs are present in trophoblast cells (Beceriklisoy *et al.* 2007, Fellows *et al.* 2012, Diessler *et al.* 2017). Biologically active inhibitors, i.e., TIMPs, by interacting with MMPs promote cell growth, inhibit angiogenesis and reveal both anti- and pro-inflammatory effects (reviewed in Stetler-Stevenson 2008, Brew & Nagase 2010). Although TIMP2 and TIMP4 are 50% identical in sequence, they still bear differences in specificity to MMPs (Brew & Nagase 2010). *In vitro* studies with human endometrial cells have shown that TIMPs are positively associated with the decidualization process and cell migration (Graham *et al.* 2017). Interestingly, *TIMP2* expression can be induced by P4 (Imada *et al.* 1994, Jo *et al.* 2015). We have previously shown the ability of canine embryos to increase uterine *TIMP2* expression prior to implantation (Graubner *et al.* 2017*a*). During canine labor also, the placental presence of *TIMP2* has been confirmed at the mRNA

level (Fellows *et al.* 2012). It is assumed that interference with TIMPs functions can lead to placental retention and SIPS in dogs, or to spontaneous early pregnancy failure and *placenta accreta* in humans (Al-Bassam *et al.* 1981, Ke *et al.* 2006, Nissi *et al.* 2013).

In addition to the previously reported increase in mRNA expression of *TIMP2* (Graubner *et al.* 2017*a*), here also, elevated TIMP4 expression was observed in the pre-implantation canine uterus. The stimulatory effect on gene expression was supported by IHC results in which free-floating embryos appeared to increase signals for TIMP2 in both superficial and deep uterine glands. Following implantation, a gradual increase was noted for both TIMPs (i.e. *TIMP2* and *-4*) in uterine and placental compartments.

An important finding from our study is the strong presence of TIMP2 and -4 in the endometrial connective tissue layer separating the superficial from deep glands. In a healthy pregnancy, trophoblast invasion is stopped at this bordering protective structure. Additionally, uterine and placental TIMPs were localized around blood vessels. Thus, it is plausible to assume that both proteins are involved in the protection of maternal tissue from trophoblast invasion. Within the placenta, TIMP2 appeared more abundantly represented than TIMP4. Both factors were, however, localized in trophoblast cells, which appears to reflect an auto-/paracrine feedback loop acting within these cells, possibly controlling own proteolytic function of trophoblast and protecting the maternal tissues from excessive invasion. Although it is not known for the dog if TIMP2 and TIMP4 interact with active MMP2 and MMP9, our findings (i.e., the gradual increase of *TIMP2*, *TIMP4* and *ECM1*) indicate that MMP-mediated-trophoblast invasion might be balanced by these factors. In this regard, future studies should be considered, investigating for example the expression of MMPs and their regulators within excessive trophoblast invasion leading to conditions such as SIPS.

Conclusions

In this study, we investigated the modulation of uterine and placental ECM in defined stages of canine pregnancy. Based on the presented results, it seems that the primary goal of the early embryo–maternal communication is the regulation of trophoblast invasion, and the proliferative and adhesive functions of the uterus. Following trophoblast attachment, the ECM is strongly modulated reflecting dynamic feto–maternal interactions during establishment of the canine endotheliochorial placenta.

The role of the endometrial connective tissue layer as an active, and not only physical but also a biochemical, barrier protecting maternal tissues from unrestrained trophoblast invasion, is strongly implied. This is supported by the abundant local expression of ECM components potentially actively involved in modulating the invasiveness of fetal cells.

Regulatory mechanisms involved in modulation of ECM components during establishment and maintenance of gestation modes characteristic of invasive placentation types, appear to be similar to what has been described for other mammalian species exhibiting similar or even more intense invasion by trophoblast (hemochorial placentation type). In particular, TIMPs and ECM1 appear to be plausible candidates involved in regulating the establishment and termination of pregnancy in the dog. With this, the dog could provide an interesting model for investigating placental functions in other species, e.g. in humans in whom *placenta accreta* appears to share several similarities with canine SIPS.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-17-0761.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. All authors read and approved the final version of the manuscript.

Funding

This work was supported by the Swiss National Science Foundation (SNSF) research grant number 31003A_160251 to M P K.

Author contribution statement

F R G: involved in developing the concept of the study, experimental design, generating data, analysis and interpretation of data and writing of the manuscript. A B: knowledge transfer, critical discussion of data, editing of the manuscript. S A and I K: knowledge transfer, critical discussion of data, collection of tissue material. M P K: designed and supervised the project, involved in interpretation of the data, drafting and revising the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The technical expertise and contribution of Elisabeth Högger is gratefully appreciated. Authors are grateful to Dr Barry Bavister for careful editing of the manuscript and to Prof. Dr Bernd Hoffmann, Justus-Liebig University Giessen, Germany and his team for providing the tissue material. Part of the laboratory work was performed using the logistics at the Center for Clinical Studies, Vetsuisse Faculty, University of Zurich.

References

Abedin M & King N 2010 Diverse evolutionary paths to cell adhesion. *Trends in Cell Biology* **20** 734–742. (https://doi.org/10.1016/j.tcb.2010.08.002)

Al-Bassam MA, Thomson RG & O'Donnell L 1981 Involution abnormalities in the postpartum uterus of the bitch. *Veterinary Pathology* **18** 208–218. (https://doi.org/10.1177/030098588101800208)

Aplin JD, Charlton AK & Ayad S 1988 An immunohistochemical study of human endometrial extracellular matrix during the menstrual cycle and first trimester of pregnancy. *Cell and Tissue Research* **253** 231–240. (https://doi.org/10.1007/BF00221758)

Attupuram NM, Kumaresan A, Narayanan K & Kumar H 2016 Cellular and molecular mechanisms involved in placental separation in the bovine: a review. *Molecular Reproduction and Development* **83** 287–297. (https://doi.org/10.1002/mrd.22635)

Aumailley M 2013 The laminin family. *Cell Adhesion and Migration* **7** 48–55. (https://doi.org/10.4161/cam.22826)

Bazer FW 2015 History of maternal recognition of pregnancy. *Advances in Anatomy, Embryology and Cell Biology* **216** 5–25.

Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC & Bayless K 2010 Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Molecular Human Reproduction* **16** 135–152. (https://doi.org/10.1093/molehr/gap095)

Beceriklisoy HB, Walter I, Schafer-Somi S, Miller I, Kanca H, Izgur H & Aslan S 2007 Matrix metalloproteinase (MMP)-2 and MMP-9 activity in the canine uterus before and during placentation. *Reproduction in Domestic Animals* **42** 654–659. (https://doi.org/10.1111/j.1439-0531.2006.00838.x)

Boos A 2000 Immunohistochemical assessment of collagen types I, III, IV and VI in biopsy samples of the bovine uterine wall collected during the oestrous cycle. *Cells Tissues Organs* **167** 225–238. (https://doi.org/10.1159/000016799)

Boos A, Stelljes A & Kohtes J 2003 Collagen types I, III and IV in the placentome and interplacentomal maternal and fetal tissues in normal cows and in cattle with retention of fetal membranes. *Cells Tissues Organs* **174** 170–183. (https://doi.org/10.1159/000072720)

Brew K & Nagase H 2010 The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. *Biochimica et Biophysica Acta* **1803** 55–71. (https://doi.org/10.1016/j.bbamcr.2010.01.003)

Brown JJ & Papaioannou VE 1992 Distribution of hyaluronan in the mouse endometrium during the periimplantation period of pregnancy. *Differentiation* **52** 61–68. (https://doi.org/10.1111/j.1432-0436.1992.tb00500.x)

Bruzzone R, White TW & Paul DL 1996 Connections with connexins: the molecular basis of direct intercellular signaling. *European Journal of Biochemistry* **238** 1–27. (https://doi.org/10.1111/j.1432-1033.1996.0001q.x)

Bukowska D, Kempisty B, Jackowska M, Wozna M, Antosik P, Piotrowska H & Jaskowski JM 2011 Analysis of integrins and vascular endothelial growth factor isoforms mRNA expression in the canine uterus during perimplantation period. *Polish Journal of Veterinary Sciences* **14** 253–258.

Cabrol D, Dallot E, Cedard L & Sureau C 1985 Pregnancy-related changes in the distribution of glycosaminoglycans in the cervix and corpus of the human uterus. *European Journal of Obstetrics and Gynecology and Reproductive Biology* **20** 289–295. (https://doi.org/10.1016/0028-2243(85)90139-X)

Cha J, Sun X Dey SK 2012 Mechanisms of implantation: strategies for successful pregnancy. *Nature Medicine* **18** 1754–1767.

Church HJ, Richards AJ & Aplin JD 1997 Laminins in decidua, placenta and choriocarcinoma cells. *Placenta* **18** 143–162. (https://doi.org/10.1016/S0143-4004(97)80085-9)

Clark DE, Hurst PR, McLennan IS & Myers DB 1993 Immunolocalization of collagen type I and laminin in the uterus on days 5 to 8 of embryo implantation in the rat. *Anatomical Record* **237** 8–20. (https://doi.org/10.1002/ar.1092370103)

Cohen M, Meisser A & Bischof P 2006 Metalloproteinases and human placental invasiveness. *Placenta* **27** 783–793. (https://doi.org/10.1016/j.placenta.2005.08.006)

Concannon PW 1993 Biology of gonadotrophin secretion in adult and prepubertal female dogs. *Journal of Reproduction and Fertility Supplement* **47** 3–27.

Concannon PW, McCann JP & Temple M 1989 Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *Journal of Reproduction and Fertility Supplement* **39** 3–25.

Cronier L, Defamie N, Dupays L, Theveniau-Ruissy M, Goffin F, Pointis G & Malassine A 2002 Connexin expression and gap junctional intercellular communication in human first trimester trophoblast. *Molecular Human Reproduction* **8** 1005–1013. (https://doi.org/10.1093/molehr/8.11.1005)

Curry TE Jr & Osteen KG 2001 Cyclic changes in the matrix metalloproteinase system in the ovary and uterus. *Biology of Reproduction* **64** 1285–1296. (https://doi.org/10.1095/biolreprod64.5.1285)

da Anunciacao A, Mess AM, Orechio D, Aguiar BA, Favaron PO & Miglino MA 2017 Extracellular matrix in epitheliochorial, endotheliochorial and haemochorial placentation and its potential application for regenerative medicine. *Reproduction in Domestic Animals* **52** 3–15. (https://doi.org/10.1111/rda.13067)

Damsky C, Sutherland A & Fisher S 1993 Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation. *FASEB Journal* **7** 1320–1329. (https://doi.org/10.1096/fasebj.7.14.8224605)

Dempsey EW & Wislocki GB 1956 Electron microscopic observations on the placenta of the cat. *Journal of Biophysical and Biochemical Cytology* **2** 743–754. (https://doi.org/10.1083/jcb.2.6.743)

Diessler M, Ventureira M, Hernandez R, Sobarzo C, Casas L, Barbeito C & Cebral E 2017 Differential expression and activity of matrix metalloproteinases 2 and 9 in canine early placenta. *Reproduction in Domestic Animals* **52** 35–43. (https://doi.org/10.1111/rda.12791)

Eiler H & Hopkins FM 1992 Bovine retained placenta: effects of collagenase and hyaluronidase on detachment of placenta. *Biology of Reproduction* **46** 580–585. (https://doi.org/10.1095/biolreprod46.4.580)

Eiler H & Hopkins FM 1993 Successful treatment of retained placenta with umbilical cord injections of collagenase in cows. *Journal of the American Veterinary Medical Association* **203** 436–443.

Engvall E, Earwicker D, Haaparanta T, Ruoslahti E & Sanes JR 1990 Distribution and isolation of four laminin variants; tissue restricted distribution of heterotrimers assembled from five different subunits. *Cell Regulation* **1** 731–740.

Evans WH & Martin PE 2002 Gap junctions: structure and function (review). *Molecular Membrane Biology* **19** 121–136. (https://doi.org/10.1080/09687680210139839)

Fazleabas AT, Bell SC, Fleming S, Sun J & Lessey BA 1997 Distribution of integrins and the extracellular matrix proteins in the baboon endometrium during the menstrual cycle and early pregnancy. *Biology of Reproduction* **56** 348–356. (https://doi.org/10.1095/biolreprod56.2.348)

Fellows EJ, Hazzard TM & Kutzler MA 2012 Gene expression in preterm, pre-labour and parturient canine placenta. *Reproduction in Domestic Animals* **47** (Supplement 6) 182–185. (https://doi.org/10.1111/rda.12021)

Franczyk M, Lopucki M, Stachowicz N, Morawska D & Kankofer M 2017 Extracellular matrix proteins in healthy and retained placentas, comparing hemochorial and synepitheliochorial placentas. *Placenta* **50** 19–24. (https://doi.org/10.1016/j.placenta.2016.12.014)

Fujimoto N, Terlizzi J, Aho S, Brittingham R, Fertala A, Oyama N, McGrath JA & Uitto J 2006 Extracellular matrix protein 1 inhibits the activity of matrix metalloproteinase 9 through high-affinity protein/protein interactions. *Experimental Dermatology* **15** 300–307. (https://doi.org/10.1111/j.0906-6705.2006.00409.x)

Goldman-Wohl D & Yagel S 2002 Regulation of trophoblast invasion: from normal implantation to pre-eclampsia. *Molecular and Cellular Endocrinology* **187** 233–238. (https://doi.org/10.1016/S0303-7207(01)00687-6)

Graham A, Holbert J & Nothnick WB 2017 miR-181b-5p modulates cell migratory proteins, tissue inhibitor of metalloproteinase 3, and annexin A2 during in vitro decidualization in a human endometrial stromal cell line. *Reproductive Sciences* **24** 1264–1274. (https://doi.org/10.1177/1933719116682877)

Gram A, Buchler U, Boos A, Hoffmann B & Kowalewski MP 2013 Biosynthesis and degradation of canine placental prostaglandins: prepartum changes in expression and function of prostaglandin F2alpha-

synthase (PGFS, AKR1C3) and 15-hydroxyprostaglandin dehydrogenase (HPGD). *Biology of Reproduction* **89** 2.

Graubner FR, Gram A, Kautz E, Bauersachs S, Aslan S, Agaoglu AR, Boos A & Kowalewski MP 2017a Uterine responses to early pre-attachment embryos in the domestic dog and comparisons with other domestic animal species. *Biology of Reproduction* **97** 197–216.

Graubner FR, Reichler IM, Rahman NA, Payan-Carreira R, Boos A & Kowalewski MP 2017b Decidualization of the canine uterus: From early until late gestational in vivo morphological observations, and functional characterization of immortalized canine uterine stromal cell lines. *Reproduction in Domestic Animals* **52** (Supplement 2) 137–147. (https://doi.org/10.1111/rda.12849)

Grinnell F, Head JR & Hoffpauir J 1982 Fibronectin and cell shape in vivo: studies on the endometrium during pregnancy. *Journal of Cell Biology* **94** 597–606. (https://doi.org/10.1083/jcb.94.3.597)

Grummer R, Chwalisz K, Mulholland J, Traub O & Winterhager E 1994 Regulation of connexin26 and connexin43 expression in rat endometrium by ovarian steroid hormones. *Biology of Reproduction* **51** 1109–1116. (https://doi.org/10.1095/biolreprod51.6.1109)

Grummer R, Reuss B & Winterhager E 1996 Expression pattern of different gap junction connexins is related to embryo implantation. *International Journal of Developmental Biology* **40** 361–367.

Grummer R, Hewitt SW, Traub O, Korach KS & Winterhager E 2004 Different regulatory pathways of endometrial connexin expression: preimplantation hormonal-mediated pathway versus embryo implantation-initiated pathway. *Biology of Reproduction* **71** 273–281. (https://doi.org/10.1095/biolreprod.103.024067)

Hannan NJ & Salamonsen LA 2008 CX3CL1 and CCL14 regulate extracellular matrix and adhesion molecules in the trophoblast: potential roles in human embryo implantation. *Biology of Reproduction* **79** 58–65. (https://doi.org/10.1095/biolreprod.107.066480)

Haouzi D, Dechaud H, Assou S, Monzo C, de Vos J & Hamamah S 2011 Transcriptome analysis reveals dialogues between human trophectoderm and endometrial cells during the implantation period. *Human Reproduction* **26** 1440–1449. (https://doi.org/10.1093/humrep/der075)

Hjelm AM, Barchan K, Malmstrom A & Ekman-Ordeberg GE 2002 Changes of the uterine proteoglycan distribution at term pregnancy and during labour. *European Journal of Obstetrics and Gynecology and Reproductive Biology* **100** 146–151. (https://doi.org/10.1016/S0301-2115(01)00476-6)

Hoffmann B, Hoveler R, Nohr B & Hasan SH 1994 Investigations on hormonal changes around parturition in the dog and the occurrence of pregnancy-specific non-conjugated oestrogens. *Experimental and Clinical Endocrinology* **102** 185–189. (https://doi.org/10.1055/s-0029-1211280)

Hubmacher D & Apte SS 2013 The biology of the extracellular matrix: novel insights. *Current Opinion in Rheumatology* **25** 65–70. (https://doi.org/10.1097/BOR.0b013e32835b137b)

Imada K, Ito A, Itoh Y, Nagase H & Mori Y 1994 Progesterone increases the production of tissue inhibitor of metalloproteinases-2 in rabbit uterine cervical fibroblasts. *FEBS Letters* **341** 109–112. (https://doi.org/10.1016/0014-5793(94)80250-5)

Jahn E, Classen-Linke I, Kusche M, Beier HM, Traub O, Grummer R & Winterhager E 1995 Expression of gap junction connexins in the human endometrium throughout the menstrual cycle. *Human Reproduction* **10** 2666–2670. (https://doi.org/10.1093/oxfordjournals.humrep.a135764)

Jo YS, Lee GS, Nam SY & Kim SJ 2015 Progesterone inhibits leptin-induced invasiveness of BeWo cells. *International Journal of Medical Sciences* **12** 773–779. (https://doi.org/10.7150/ijms.11610)

Johnson GA, Burghardt RC, Bazer FW & Spencer TE 2003 Osteopontin: roles in implantation and placentation. *Biology of Reproduction* **69** 1458–1471. (https://doi.org/10.1095/biolreprod.103.020651)

Johnson ML, Redmer DA, Reynolds LP & Grazul-Bilska AT 2017 Gap junctional connexin messenger RNA expression in the ovine uterus and placenta: effects of estradiol-17beta-treatment, early pregnancy stages, and embryo origin. *Domestic Animal Endocrinology* **58** 104–112. (https://doi.org/10.1016/j.domaniend.2016.09.004)

Kaloglu C & Onarlioglu B 2010 Extracellular matrix remodelling in rat endometrium during early pregnancy: the role of fibronectin and laminin. *Tissue and Cell* **42** 301–306. (https://doi.org/10.1016/j.tice.2010.07.004)

Kautz E, Gram A, Aslan S, Ay SS, Selcuk M, Kanca H, Koldas E, Akal E, Karakas K, Findik M *et al*. 2014 Expression of genes involved in the embryo-maternal interaction in the early-pregnant canine uterus. *Reproduction* **147** 703–717. (https://doi.org/10.1530/REP-13-0648)

Kautz E, de Carvalho Papa P, Reichler IM, Gram A, Boos A & Kowalewski MP 2015 In vitro decidualisation of canine uterine stromal cells. *Reproductive Biology and Endocrinology* **13** 85. (https://doi.org/10.1186/s12958-015-0066-4)

Ke Y, Lu JH, Yang BL, Guo HQ, Ma QY, Zhu H, Shu HM & Li DJ 2006 [Involvement of matrix metalloproteinase-2, -9, and tissue inhibitors of metalloproteinase-1, 2 in occurrence of the accrete placenta]. *Zhonghua Fu Chan Ke Za Zhi* **41** 311–314.

Kisalus LL, Herr JC & Little CD 1987 Immunolocalization of extracellular matrix proteins and collagen synthesis in first-trimester human decidua. *Anatomical Record* **218** 402–415. (https://doi.org/10.1002/ar.1092180408)

Kleinman HK, Cannon FB, Laurie GW, Hassell JR, Aumailley M, Terranova VP, Martin GR & DuBois-Dalcq M 1985 Biological activities of laminin. *Journal of Cellular Biochemistry* **27** 317–325. (https://doi.org/10.1002/jcb.240270402)

Korgun ET, Cayli S, Asar M & Demir R 2007 Distribution of laminin, vimentin and desmin in the rat uterus during initial stages of implantation. *Journal of Molecular Histology* **38** 253–260. (https://doi.org/10.1007/s10735-007-9095-4)

Korhonen M & Virtanen I 2001 Immunohistochemical localization of laminin and fibronectin isoforms in human placental villi. *Journal of Histochemistry and Cytochemistry* **49** 313–322. (https://doi.org/10.1177/002215540104900305)

Kowalewski MP 2014 Luteal regression vs prepartum luteolysis: regulatory mechanisms governing canine corpus luteum function. *Reproductive Biology* **14** 89–102. (https://doi.org/10.1016/j.repbio.2013.11.004)

Kowalewski MP, Mason JI, Howie AF, Morley SD, Schuler G & Hoffmann B 2006a Characterization of the canine 3beta-hydroxysteroid dehydrogenase and its expression in the corpus luteum during diestrus. *Journal of Steroid Biochemistry and Molecular Biology* **101** 254–262. (https://doi.org/10.1016/j.jsbmb.2006.06.029)

Kowalewski MP, Schuler G, Taubert A, Engel E & Hoffmann B 2006b Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. *Theriogenology* **66** 1423–1430. (https://doi.org/10.1016/j.theriogenology.2006.01.039)

Kowalewski MP, Beceriklisoy HB, Aslan S, Agaoglu AR & Hoffmann B 2009 Time related changes in luteal prostaglandin synthesis and steroidogenic capacity during pregnancy, normal and antiprogestin induced luteolysis in the bitch. *Animal Reproduction Science* **116** 129–138. (https://doi.org/10.1016/j.anireprosci.2008.12.011)

Kowalewski MP, Beceriklisoy HB, Pfarrer C, Aslan S, Kindahl H, Kucukaslan I & Hoffmann B 2010 Canine placenta: a source of prepartal prostaglandins during normal and antiprogestin-induced parturition. *Reproduction* **139** 655–664. (https://doi.org/10.1530/REP-09-0140)

Kowalewski MP, Meyer A, Hoffmann B, Aslan S & Boos A 2011 Expression and functional implications of peroxisome proliferator-activated receptor gamma (PPARgamma) in canine reproductive tissues during normal pregnancy and parturition and at antiprogestin induced abortion. *Theriogenology* **75** 877–886. (https://doi.org/10.1016/j.theriogenology.2010.10.030)

Kumar NM & Gilula NB 1996 The gap junction communication channel. *Cell* **84** 381–388. (https://doi.org/10.1016/S0092-8674(00)81282-9)

Laird DW 2006 Life cycle of connexins in health and disease. *Biochemical Journal* **394** 527–543. (https://doi.org/10.1042/BJ20051922)

Lala PK & Nandi P 2016 Mechanisms of trophoblast migration, endometrial angiogenesis in preeclampsia: the role of decorin. *Cell Adhesion and Migration* **10** 111–125. (https://doi.org/10.1080/19336918.2015.1106669)

Laws MJ, Taylor RN, Sidell N, DeMayo FJ, Lydon JP, Gutstein DE, Bagchi MK & Bagchi IC 2008 Gap junction communication between uterine stromal cells plays a critical role in pregnancy-associated neovascularization and embryo survival. *Development* **135** 2659–2668. (https://doi.org/10.1242/dev.019810)

Lee K-M, Nam K, Oh S, Lim J, Kim Y-P, Lee JW, Yu J-H, Ahn S-H, Kim S-B, Noh D-Y *et al*. 2014 Extracellular matrix protein 1 regulates cell proliferation and trastuzumab resistance through activation of epidermal growth factor signaling. *Breast Cancer Research* **16** 479. (https://doi.org/10.1186/s13058-014-0479-6)

Leoni P, Carli F & Halliday D 1990 Collagen in the pregnant and non pregnant uterus. *Clinical Science* **79** 10P–11P.

Menon R, Bonney EA, Condon J, Mesiano S & Taylor RN 2016 Novel concepts on pregnancy clocks and alarms: redundancy and synergy in human parturition. *Human Reproduction Update* **22** 535–560. (https://doi.org/10.1093/humupd/dmw022)

Montazeri M, Sanchez-Lopez JA, Caballero I, Maslehat Lay N, Elliott S, Lopez-Martin S, Yanez-Mo M & Fazeli A 2015 Activation of Toll-like receptor 3 reduces actin polymerization and adhesion molecule expression in endometrial cells, a potential mechanism for viral-induced implantation failure. *Human Reproduction* **30** 893–905. (https://doi.org/10.1093/humrep/deu359)

Nair RR, Jain M & Singh K 2011 Reduced expression of gap junction gene connexin 43 in recurrent early pregnancy loss patients. *Placenta* **32** 619–621. (https://doi.org/10.1016/j.placenta.2011.05.010)

Nissi R, Talvensaari-Mattila A, Kotila V, Niinimaki M, Jarvela I & Turpeenniemi-Hujanen T 2013 Circulating matrix metalloproteinase MMP-9 and MMP-2/TIMP-2 complex are associated with spontaneous early pregnancy failure. *Reproductive Biology and Endocrinology* **11** 2. (https://doi.org/10.1186/1477-7827-11-2)

Niswender GD, Juengel JL, Silva PJ, Rollyson MK & McIntush EW 2000 Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews* **80** 1–29. (https://doi.org/10.1152/physrev.2000.80.1.1)

Oliver C, Montes MJ, Galindo JA, Ruiz C & Olivares EG 1999 Human decidual stromal cells express alpha-smooth muscle actin and show ultrastructural similarities with myofibroblasts. *Human Reproduction* **14** 1599–1605.

Oliveira GBd, Vale AMd, Santos ACd, Moura CEBd, Rocha HAdO & Oliveira MFd 2015 Composition and significance of glycosaminoglycans in the uterus and placenta of mammals. *Brazilian Archives of Biology and Technology* **58** 512–520. (https://doi.org/10.1590/S1516-8913201500281)

Paidas MJ, Krikun G, Huang SJ, Jones R, Romano M, Annunziato J & Barnea ER 2010 A genomic and proteomic investigation of the impact of preimplantation factor on human decidual cells. *American Journal of Obstetrics and Gynecology* **202** 459.e451–459.e458.

Sagsoz H, Liman N, Saruhan BG & Kucukaslan I 2014 The expression of epidermal growth factor receptors and their ligands (epidermal growth factor, neuregulin, amphiregulin) in the bitch uterus during the estrus cycle. *Animal Reproduction Science* **147** 161–179. (https://doi.org/10.1016/j.anireprosci.2014.04.005)

Seshagiri PB, Sen Roy S, Sireesha G & Rao RP 2009 Cellular and molecular regulation of mammalian blastocyst hatching. *Journal of Reproductive Immunology* **83** 79–84. (https://doi.org/10.1016/j.jri.2009.06.264)

Short RV 1969. Implantation and the maternal recognition of pregnancy. In *Ciba Foundation Symposium on Foetal Autonomy*, G EW Wolstenholme & M O'Connor (eds) pp 2–31 London: Churchill.

Smith SD, Choudhury RH, Matos P, Horn JA, Lye SJ, Dunk CE, Aplin JD, Jones RL & Harris LK 2016 Changes in vascular extracellular matrix composition during decidual spiral arteriole remodeling in early human pregnancy. *Histology and Histopathology* **31** 557–571.

Smits P, Ni J, Feng P, Wauters J, Van Hul W, Boutaibi ME, Dillon PJ & Merregaert J 1997 The human extracellular matrix gene 1 (ECM1): genomic structure, cDNA cloning, expression pattern, and chromosomal localization. *Genomics* **45** 487–495. (https://doi.org/10.1006/geno.1997.4918)

Stetler-Stevenson WG 2008 Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. *Science Signaling* **1** re6.

Strauss JF 3rd 2013 Extracellular matrix dynamics and fetal membrane rupture. *Reproductive Sciences* **20** 140–153. (https://doi.org/10.1177/1933719111424454)

Sutherland AE, Calarco PG & Damsky CH 1993 Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Development* **119** 1175–1186.

Vermeirsch H, Simoens P & Lauwers H 2000 Immunohistochemical detection of the estrogen receptor-alpha and progesterone receptor in the canine pregnant uterus and placental labyrinth. *Anatomical Record* **260** 42–50. (https://doi.org/10.1002/1097-0185(20000901)260:1<42::AID-AR50>3.0.CO;2-8)

Wewer UM, Faber M, Liotta LA & Albrechtsen R 1985 Immunochemical and ultrastructural assessment of the nature of the pericellular basement membrane of human decidual cells. *Laboratory Investigation* **53** 624–633.

Wewer UM, Damjanov A, Weiss J, Liotta LA & Damjanov I 1986 Mouse endometrial stromal cells produce basement-membrane components. *Differentiation* **32** 49–58. (https://doi.org/10.1111/j.1432-0436.1986.tb00555.x)

Winterhager E, Grummer R, Jahn E, Willecke K & Traub O 1993 Spatial and temporal expression of connexin26 and connexin43 in rat endometrium during trophoblast invasion. *Developmental Biology* **157** 399–409. (https://doi.org/10.1006/dbio.1993.1144)

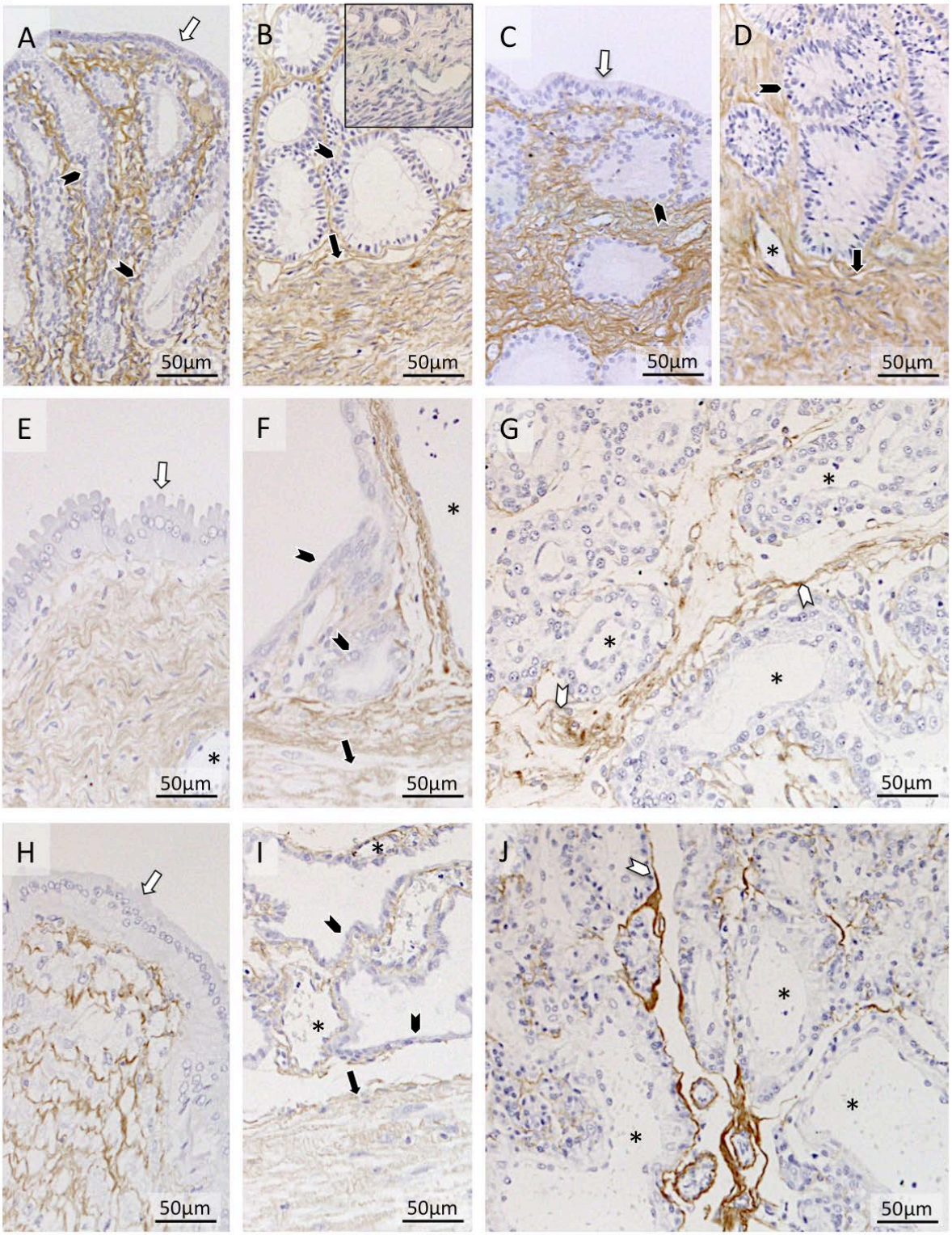
Wislocki GB & Dempsey EW 1946 Histochemical reactions in the placenta of the cat. *American Journal of Anatomy* **78** 1–45. (https://doi.org/10.1002/aja.1000780102)

Zhang C, Duan E, Cao Y, Jiang G & Zeng G 2000 Effect of 32/67 kDa laminin-binding protein antibody on mouse embryo implantation. *Journal of Reproduction and Fertility* **119** 137–142. (https://doi.org/10.1530/reprod/119.1.137)

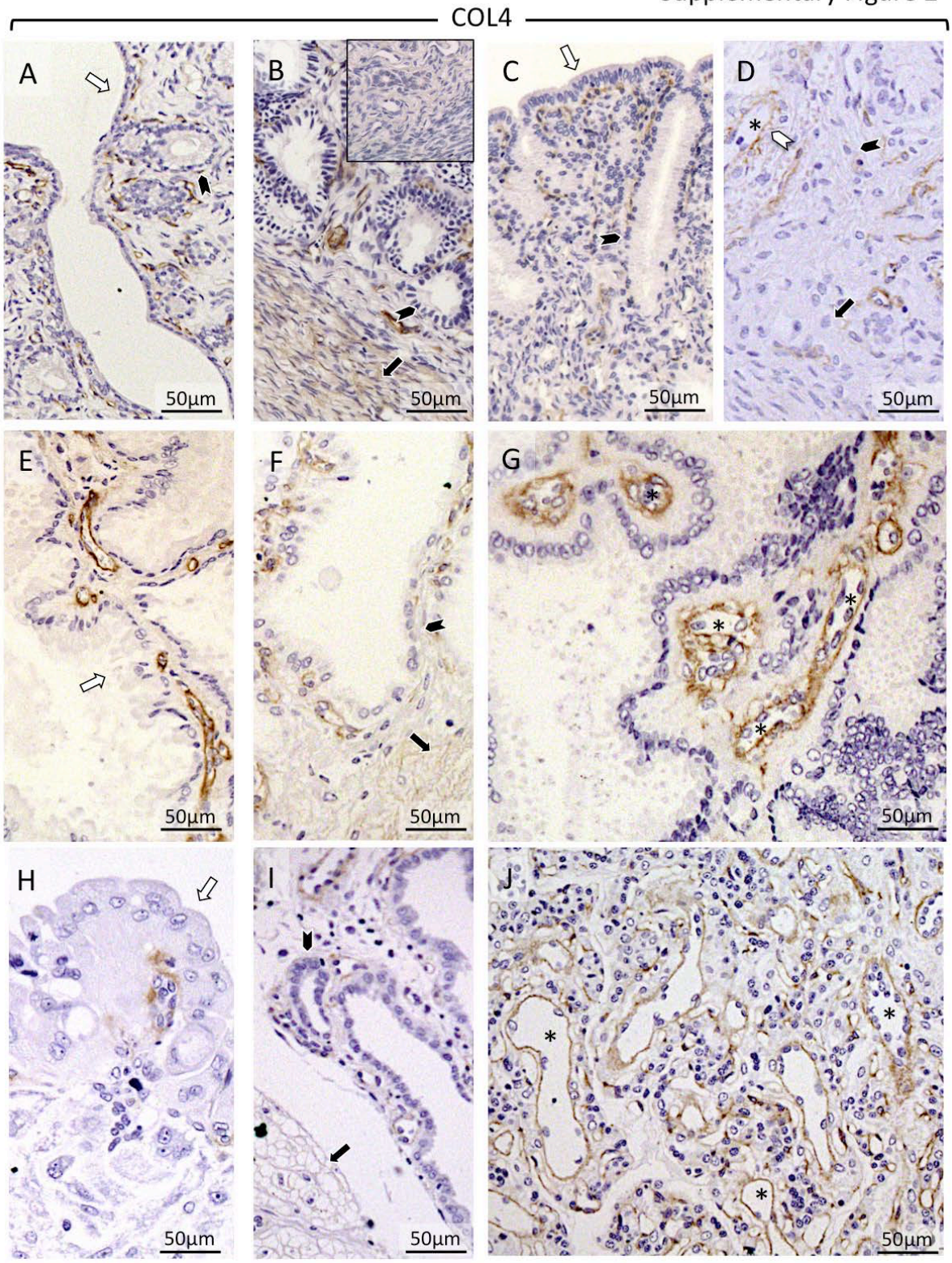
Zollinger AJ & Smith ML 2017 Fibronectin, the extracellular glue. *Matrix Biology* **60–61** 27–37. (https://doi.org/10.1016/j.matbio.2016.07.011)

Received 18 December 2017
First decision 12 January 2018
Revised manuscript received 24 January 2018
Accepted 9 February 2018

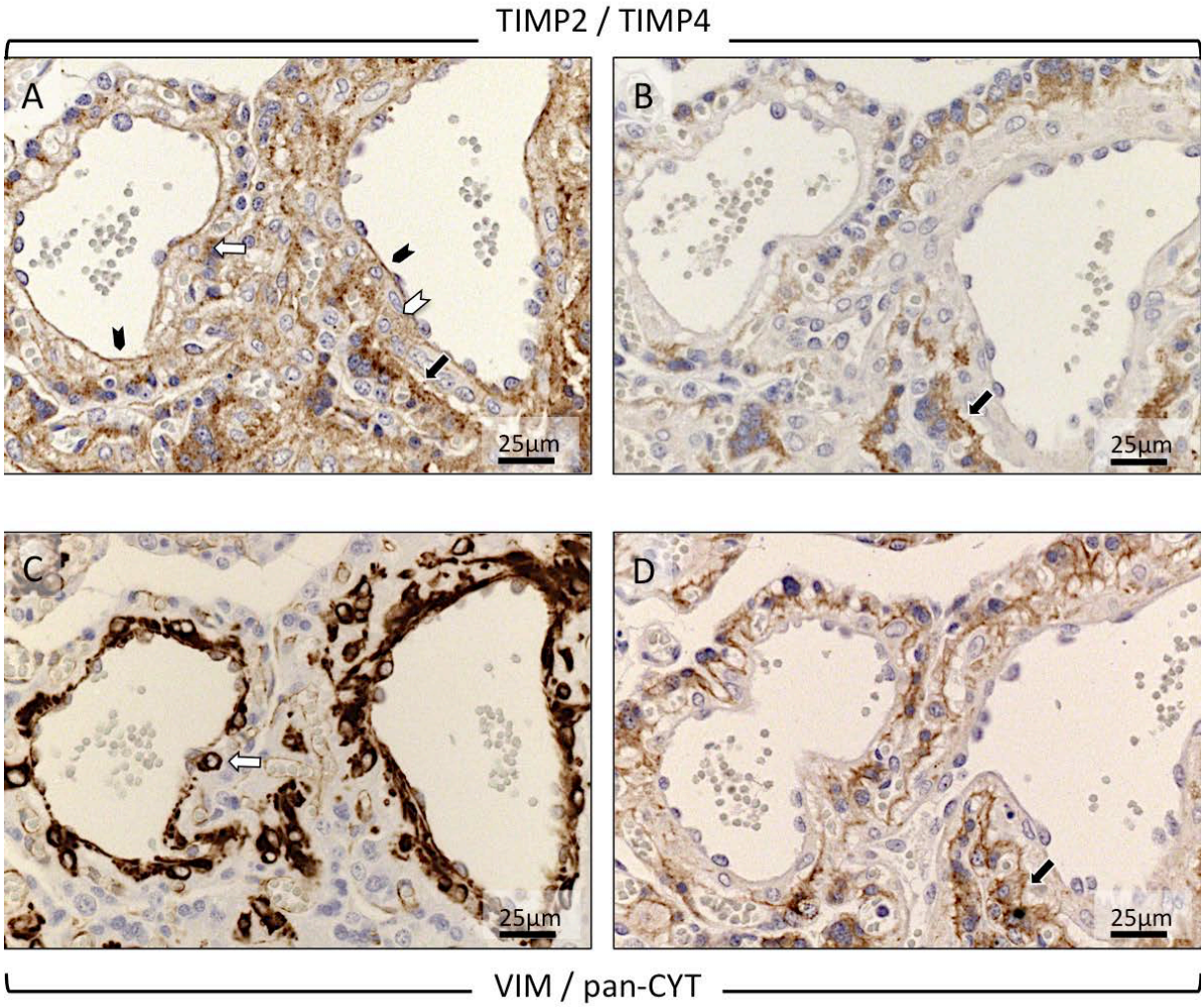
Supplementary Figure 1



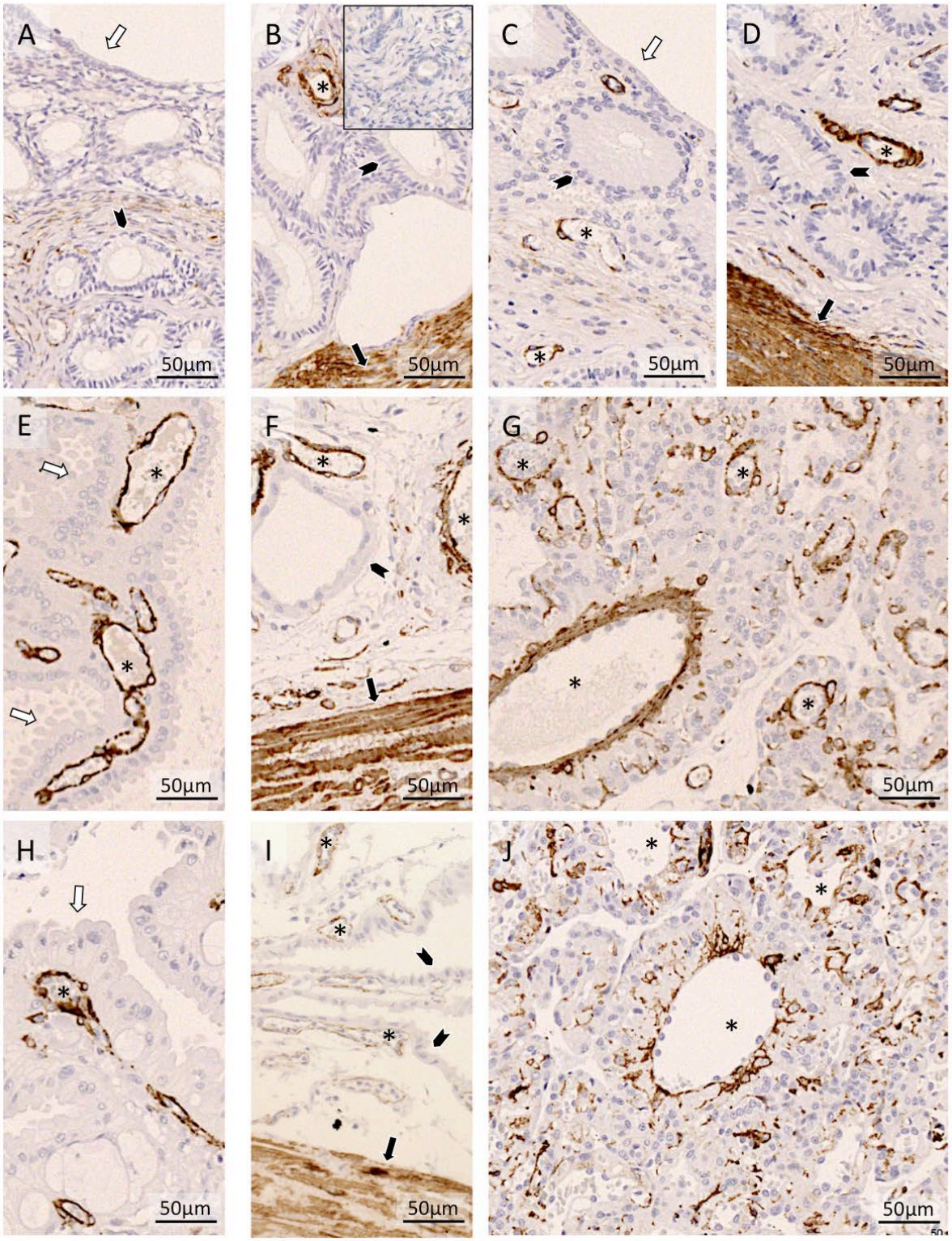
Supplementary Figure 2



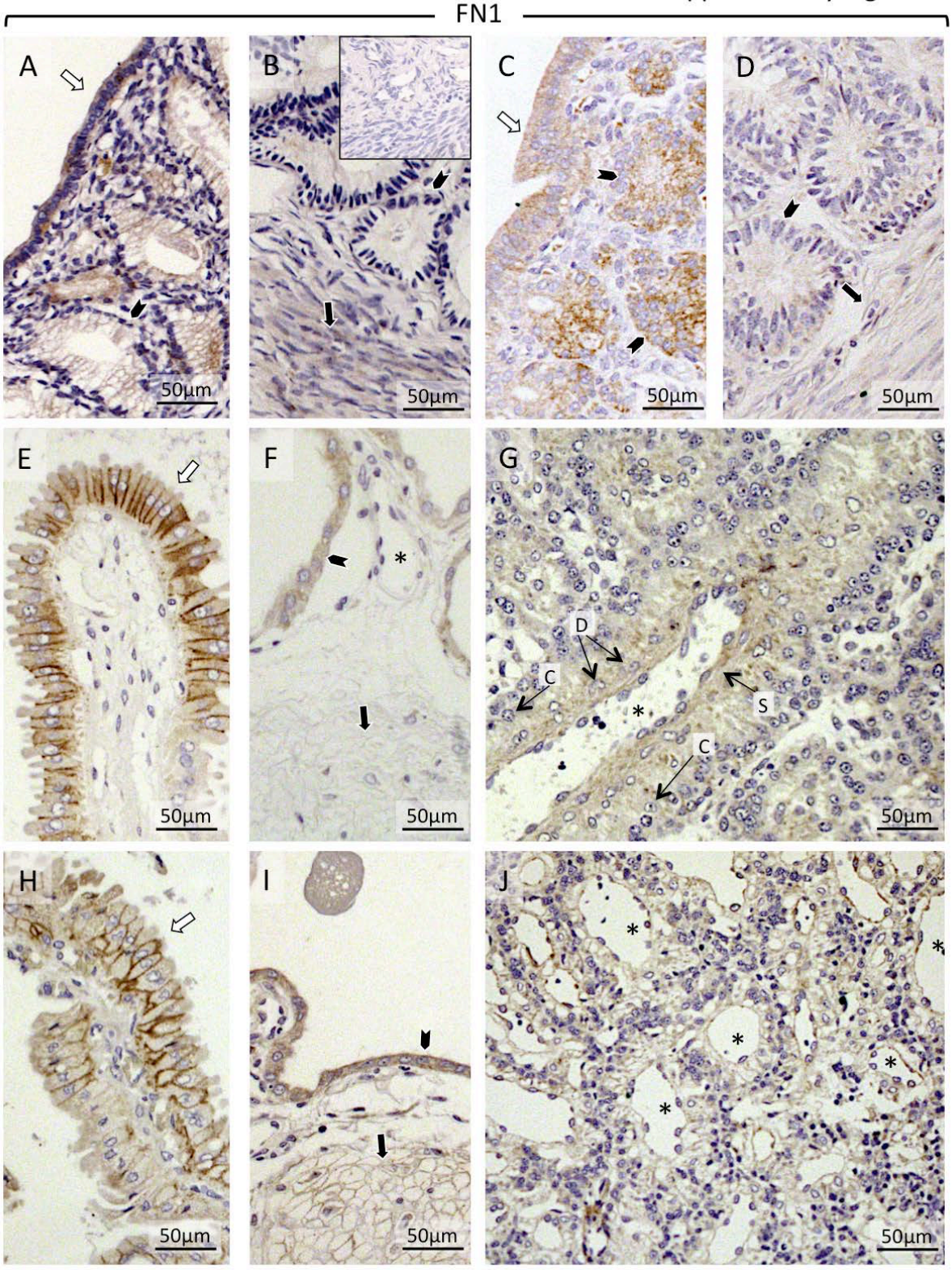
Supplementary Figure 3



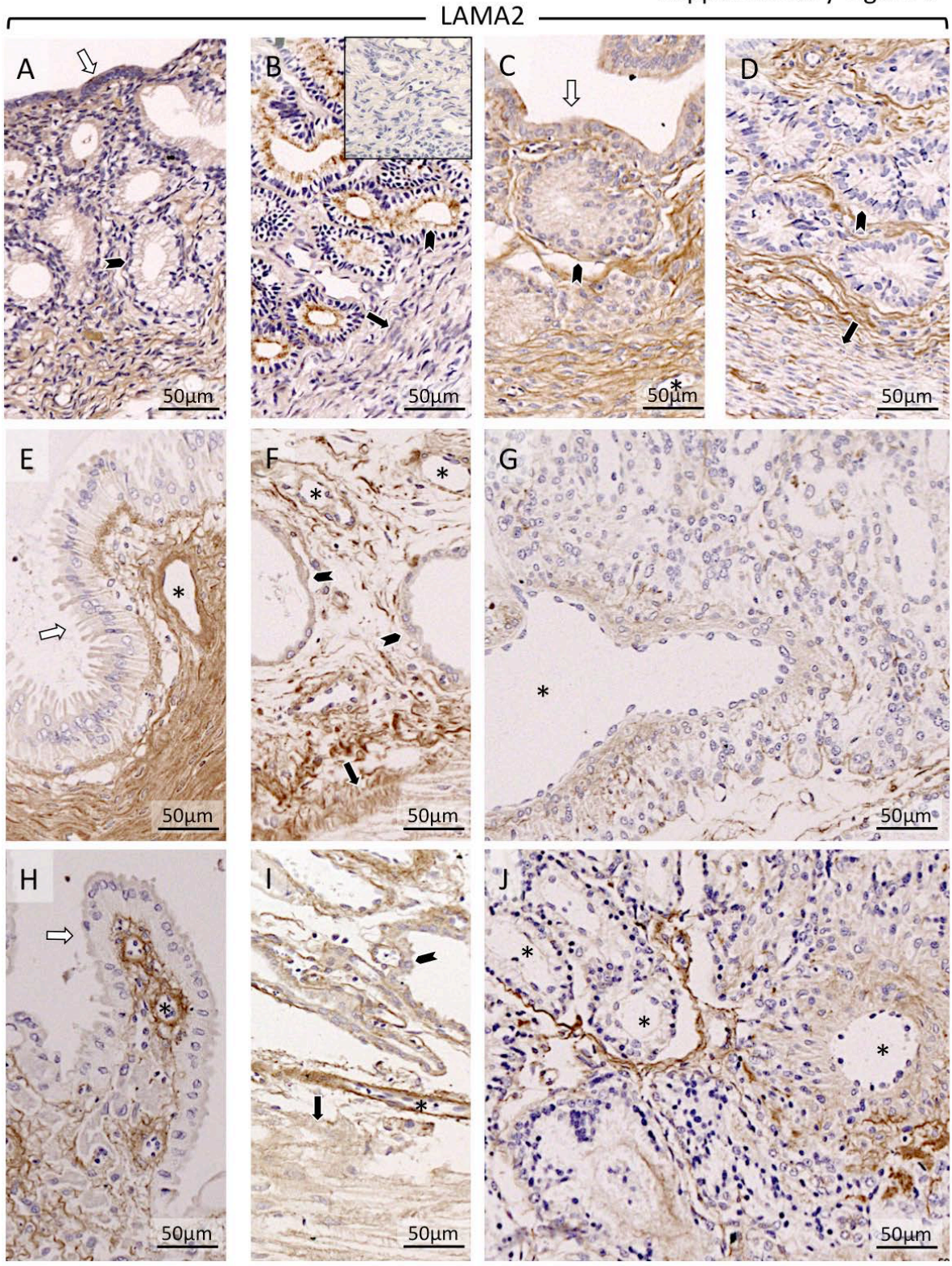
Supplementary Figure 4



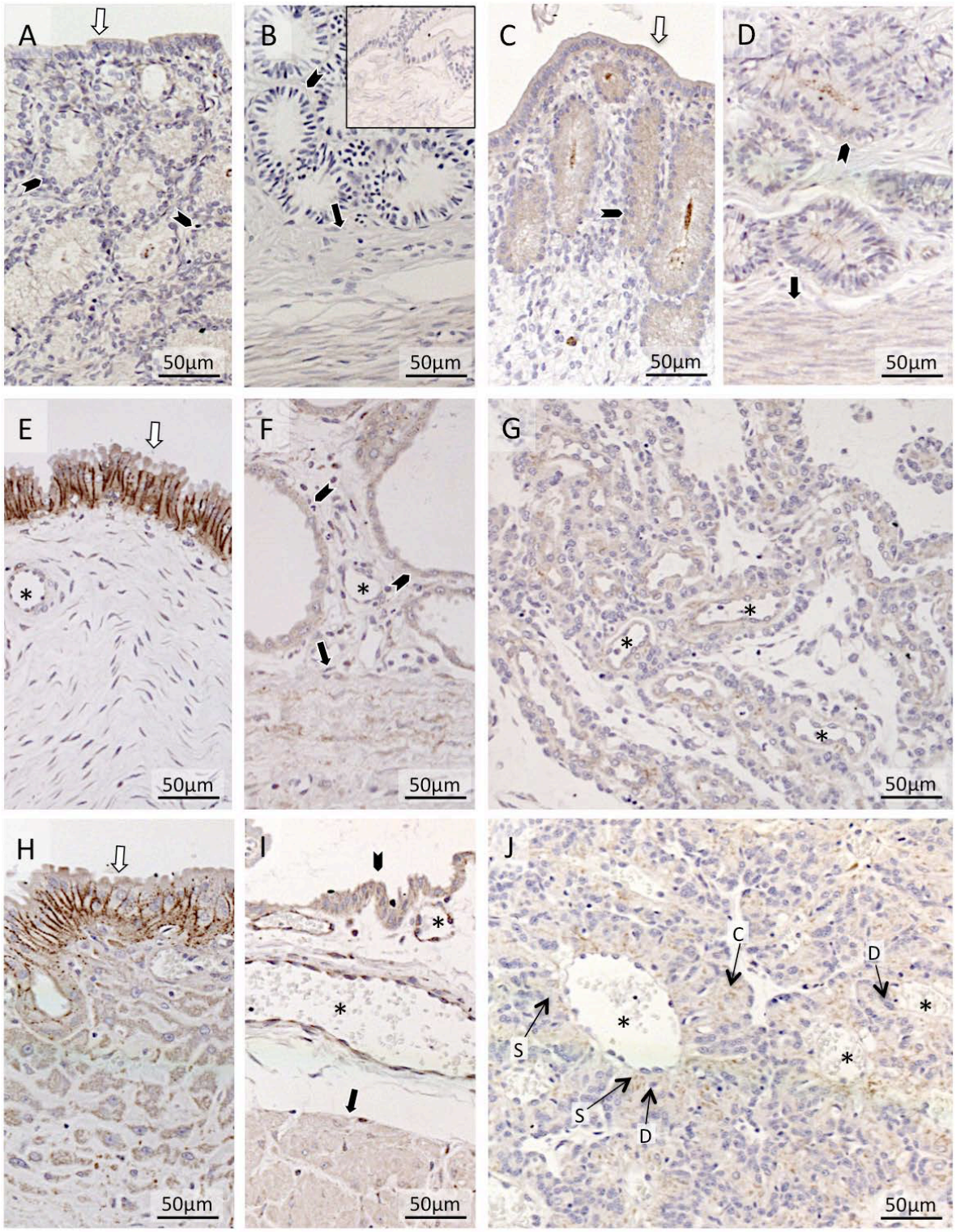
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Manuscript 3: Decidualization of the canine uterus: From early until late gestational in vivo morphological observations, and functional characterization of immortalized canine uterine stromal cell lines

Graubner FR, Reichler IM, Rahman NA, Payan-Carreira R, Boos A, Kowalewski MP.

Published in: Reprod Domest Anim. 2017 Apr;52 Suppl 2:137-147. doi:10.1111/rda.12849. Epub 2016 Nov 15. PMID:27862405

Contribution:

Felix R. Graubner was involved in the development of the concept of the study, experimental design, the laboratory part of the project, tissue processing, generating data, analysis and interpretation of data and writing of the manuscript.

DOI: 10.1111/rda.12849

ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

Decidualization of the canine uterus: From early until late gestational in vivo morphological observations, and functional characterization of immortalized canine uterine stromal cell lines

FR Graubner[1] | IM Reichler[2] | NA Rahman[3] | R Payan-Carreira[4] | A Boos[1] | MP Kowalewski[1]

[1]Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

[2]Clinic of Reproductive Medicine, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

[3]Department of Physiology, Institute of Biomedicine, University of Turku, Turku, Finland

[4]Centro de Ciência Animal e Veterinária (CECAV), Zootecnia Department, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

Correspondence
Prof. Mariusz P. Kowalewski, DVM PhD, Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.
Emails: kowalewskipl@yahoo.de or kowalewski@vetanat.uzh.ch

Funding information
Swiss National Science Foundation (SNSF), Grant/Award Number: 31003A_160251

Contents

The apparent lack of classical mechanisms for maternal recognition of pregnancy is one of the most intriguing features of canine reproduction. Consequently, similar levels of circulating luteal steroids are observed in pregnant and non-pregnant dogs. However, the early pre-implantation canine embryo locally modulates uterine responses to its presence, facilitating the successful onset of pregnancy. As a part of this interaction, the canine uterus undergoes a species-specific decidualization. Maternal stroma-derived decidual cells develop, the only cells of the canine placenta expressing progesterone receptor (PGR). There exists an acute need for an in vitro stable cell line model for canine decidualization. Therefore, herein our goal was to establish, immortalize and characterize such a cell line. We immortalized three monolayer dog uterine stromal (DUS) cell lines by stably transfecting them with SV40Tag oncogene. Cells retained their mesenchymal character for over 30 passages, as evidenced by VIMENTIN staining. Genomic incorporation of the SV40Tag protein was confirmed by immunofluorescence and Western blot analyses. Cells submitted to a classical in vitro decidualization protocol (N6,2′-O-dibutyryladenosine-3′,5′-cyclic monophosphate) revealed upregulated gene levels of selected major decidualization markers (*e.g. PRLR*, *PGR*, *IGF1*, *PTGES*). Additionally, the basic decidualization capability of PGE2 was demonstrated, revealing increased levels of, for example, *PGR* and *PRLR* gene expression, thereby implying its involvement in the progesterone-dependent decidualization in the canine uterus. In summary, our in vitro model with immortalized DUS cell line could serve as an ideal and unique model to study the underlying molecular and endocrine mechanisms of canine decidualization.

1 | INTRODUCTION

The canine (*Canis lupus familiaris*) reproductive cycle exhibits species-specific attributes that are unique among domestic animals. One of the most intriguing of these is the apparent inverse relationship between the length of pregnancy vs. that of a non-pregnant cycle. The non-pregnant cycle is characterized by an exceptionally long phase of physiological dioestrus, frequently exceeding the length of gestation (Concannon, McCann, & Temple, 1989; Steinetz, Goldsmith, Harvey, & Lust, 1989). This is due to the lack of a detectable luteolysin in the absence of pregnancy. As the non-pregnant luteal phase is similar to the pregnant one, a classical mechanism of maternal recognition of pregnancy allowing the extension of the luteal phase in pregnant animals, as known in other species, such as oestrogens produced by

the porcine trophoblast (Perry, Heap, & Amoroso, 1973) or interferon-tau (IFN-τ) produced by the early embryo in ruminants (Bazer et al., 1991; Spencer & Bazer, 2004), is missing and not required in the bitch. Another unique characteristic of canine reproductive physiology is that progesterone (P4) is exclusively produced by the corpus luteum (CL) (Concannon et al., 1989) and, at least in non-pregnant dogs, ovarian function is independent of a uterine luteolysin (Hoffmann, Hoveler, Hasan, & Failing, 1992). Interestingly, intraluteally produced prostaglandins (PGs) are utilized for CL support rather than termination of its function (Gram, Buchler, Boos, Hoffmann, & Kowalewski, 2013; Kowalewski, Beceriklisoy, Aslan, Agaoglu, & Hoffmann, 2009; Kowalewski, Fox, Gram, Boos, & Reichler, 2013; Kowalewski, Mutembei, & Hoffmann, 2008; Kowalewski, Schuler, Taubert, Engel, & Hoffmann, 2006).

Collectively, especially when the lack of an active luteolytic principle is taken into consideration, the dog appears to be an interesting animal model for studying the evolutionarily determined mechanisms of implantation and pregnancy. Nevertheless, in the dog embryo- and feto-maternal crosstalks have evolved that result in biochemical and morphological changes of the uterus. Ultimately, these changes result in a strong structural remodelling of the uterus and formation of canine decidua on the way to formation of the canine endotheliochorial placenta. Clearly, the strongest indication of decidualization is the formation of maternal stroma-derived decidual cells. These cells not only change their morphological characteristics by becoming round in shape but, together with the endothelial cells of maternal blood vessels, display biochemical features preventing them from being invaded by the foetal trophoblast. Additionally, maternal decidual cells are the only canine placental cells expressing the progesterone receptor (PGR) (Kowalewski et al., 2010; Vermeirsch, Simoens, & Lauwers, 2000). Any interference with its functioning, for example by applying an antigestagen, leads unequivocally to preterm parturition/abortion (Kowalewski et al., 2010). This underlines the immense importance of decidual cells during the establishment and maintenance of canine gestation.

The intimate and well-timed embryo-maternal communication and synchronized preparation for implantation are crucial steps towards successful pregnancy in mammals, including the dog. This led us to investigate the endocrine and biochemical mechanisms involved in canine decidualization. The effects exerted on the uterus by free-floating embryos were explored during the early, pre-implantation stage of gestation (Kautz et al., 2014). Several so-called decidualization markers were found in the early-pregnant uterus. Some of them were modulated by the presence of embryos, for example prolactin (PRL) receptor (PRLR) (but not PRL itself) and estrogen receptor alpha (ERα, ESR1), as well as members of the VEGF-system family (Gram, Hoffmann, Boos, & Kowalewski, 2015; Kautz et al., 2014; Schäfer-Somi et al., 2013). Additionally, some of the factors of the prostaglandin (PG) system were highly represented. Especially worth attention is the strong expression of PGE2 synthase (PTGES) and the two PGE2 receptors, that is PTGER2 and PTGER4 (formerly known as EP2 and EP4). Besides its strong luteotropic effects, PGE2 was shown to accelerate P4- and cAMP-mediated decidualization in humans and rodents (Brar, Frank, Kessler, Cedars, & Handwerger, 1997; Gellersen & Brosens, 2003; Kennedy & Doktorcik, 1988; Yee & Kennedy, 1993). This effect could also apply in the dog, as implied by the concomitant, abundant expression of *PTGES* in hatched free-floating embryos in contrast to unhatched ones (Kautz et al., 2014). Moreover, PTGES is also strongly present in maternal and foetal uterine and placental tissues throughout gestation (Gram et al., 2014). Thus, the pre-implantation canine uterus revealed higher expression of PTGES and the PTGER2 and PTGER4 receptors than its non-pregnant counterparts (Kautz et al., 2014). Their placental expression is predominantly targeted to the foetal trophoblast (Kautz et al., 2014). It is noteworthy that in humans PGE2 exhibits immunomodulatory but embryo-protective effects (Parhar, Yagel, & Lala, 1989).

Consequently, there is emerging evidence identifying PGE2 as an important factor involved in canine decidualization. This assumption is further strengthened by our recent observations from studies investigating the capability of canine uterine stromal cells to undergo decidualization in vitro (Kautz et al., 2015). Utilizing the cAMP-mediated approach with stromal cells isolated from naturally oestrogen-primed uteri from early dioestrus dogs, *PTGES* was strongly induced during the course of in vitro decidualization (Kautz et al., 2015). The cAMP-based protocol was chosen for that study due to its strong decidualization potential as shown previously (Brar et al., 1997; Gellersen & Brosens, 2003; Tamura et al., 2012). Along with the *PTGER2* and *PTGER4* receptors, the expression of some other decidualization-relevant factors, for example *IGF1*, *IGF2*, *PRLR* and *ERα*, was clearly detectable in these decidualized cells, but *PRL* expression was not found (Kautz et al., 2015). Due to senescence and differentiation of cells passaged (p) 2–3 times, only cells after $p = 1$ were used in that previous study (Kautz et al., 2015).

Here, to avoid shifts in biochemical behaviour, and because of the still-limited availability of tissue material, dog uterine stromal (DUS) cells were stably transfected with a plasmid vector carrying the large T-antigen (Tag) of simian vacuolating virus 40 (pSV40Tag). The capability of immortalized cells to decidualize in vitro was verified by applying the previously established cAMP-mediated protocol (Kautz et al., 2015). Additionally, given the potential role of PGE2 during canine decidualization, the expression of selected decidualization markers was investigated in PGE2-stimulated cells.

2 | MATERIAL AND METHODS

2.1 | Immunohistochemical characterization of decidual cell development in canine uterus and placenta in vivo

All experimental procedures were carried out in accordance with animal welfare legislation and approved by the respective authorities of the Justus-Liebig University Giessen (permit no. II 25.3-19c20-15c GI 18/14 and VIG3-19c-20/15c GI 18,14), Giessen, Germany, and the University of Ankara (permit no. Ankara 2006/06), Ankara, Turkey, or (samples collected at day 17 of pregnancy) were obtained from the "Hospital Veterinário do Baixo Vouga," Estrada Nacional 1, 355 - Segadães, 3750-742, Águeda, Portugal, following routine elective

ovariohysterectomy (OHE) with the expressed consent of the animals' owners.

Dogs were mated 2–3 days after ovulation (time needed for oocyte maturation and completion of the first meiotic division), as determined by vaginal cytology and by P4 measurements (>5 ng/ml in peripheral blood). The day of mating was designated as Day 0 of gestation.

The following tissue material was used for immunohistochemistry (IHC) (n = 3–6 per group): (1) uteri from early-pregnant dogs, determined by embryo flushings 10–12 days after mating (pre-attachment, embryo free-floating phase); (2) dogs corresponding to the first group, mated but subsequently determined to be non-pregnant by uterine flushings; (3) uteri collected during implantation, day 17 of pregnancy; (4) utero-placental cross sections (the whole thickness of the uterine wall, that is uterus with adjacent placenta) collected at midgestation (days 35–40 of pregnancy); and (5) utero-placental cross sections from the pre-partum luteolysis stage.

For more detailed description of our tissue sampling and processing procedures, see prior reports (Gram et al., 2013; Kautz et al., 2014; Kowalewski et al., 2010). Following collection, tissues were fixed for 24 h in 10% neutral phosphate-buffered formalin at 4°C and subsequently washed for 1 week with PBS and embedded in paraffin wax. Two consecutive histological slides were prepared from each tissue (2–3 µm in thickness). Of these, one was stained with haematoxylin and eosin (HE), and the second was used for IHC by applying our indirect immunoperoxidase method (Gram et al., 2014) with a primary, monoclonal mouse anti-VIMENTIN IgG2a (M7020; clone 3B4; dilution 1:100; Dako Schweiz AG, Baar, CH). For negative controls, either respective, irrelevant, non-immune mouse IgG (I-2000; Vector Laboratories Inc., Burlingame, CA, USA) was used at the same protein concentration as for the primary antibody, or the secondary antibody was omitted. The biotinylated secondary antibody was horse anti-mouse IgG, dilution 1:100 (BA-2000; Vector Laboratories Inc.). Signals were enhanced using the Vectastain ABC Kit (Vector Laboratories Inc.), and colour reactions were developed with the Liquid DAB+ substrate Kit (Dako Schweiz AG). Haematoxylin counterstaining was performed.

2.2 | Collection of tissues, isolation of cells and immortalization of dog uterine stromal cells

Uterine tissues from three non-pregnant mix-breed bitches, that underwent routine OHE at the Section of Small Animal Reproduction, Clinic of Reproductive Medicine, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland, were used with the expressed consent of the animals' owners. Their general and genital health was without diagnostic findings. OHE was performed during early dioestrus, 12–14 days after the clinical signs of heat had faded. The detailed protocols for sample collection, including enzymatic dissociation by collagenase and subsequent cell culture conditions, were as described previously (Kautz et al., 2015). Cells were seeded into 100 mm × 20 mm sterile polystyrene petri dishes (Corning, Amsterdam, NL). Differential adhesion time was used to separate epithelial cells from those of stromal origin by replacing the medium 1 h after seeding into the petri dish with fresh cell culture medium (DMEM-High Glucose, pH 7.2–7.4, 10% heat-inactivated FBS (Fetal Bovine Serum), 100 U/ml penicillin and 100 µg/ml streptomycin and 1% ITS (insulin–transferrin–selenium); all from Chemie Brunschwig AG, Basel, CH). Standard culture conditions were applied: 37°C, 5% CO_2 in air, in a humidified incubator. After cells reached approximately 80% confluence, they were transfected using the pSV40Tag vector (Chrusciel et al., 2011; Korzekwa, Bodek, Bukowska, Blitek, & Skarzynski, 2011). FuGENE HD transfection reagent (Roche Diagnostics) was used according to the manufacturer's protocol and as previously described (Gram et al., 2013), in a ratio of 1 µg DNA to 3.5 µl reagent. When cells reached full confluence, they were trypsinized and transferred into a culture flask (Corning), followed by further passages. Three cell lines were created, designated as DUS, DUS1 and DUS2. The morphological appearance of transfected cells was compared with those of primary uterine stromal cells using an inverted wide field microscope (DM IL LED Fluo, Leica Microsystems CMS GmbH, Wetzlar, DE).

2.3 | Characterization of cells and in vitro decidualization protocol

Each of the cell lines was characterized and used in the decidualization protocol. At least three experiments were performed with each cell line, starting with p = 7–10 (depending on the cell line). Subsequent experiments were performed after the next 2 passages. To verify that cells did not change their biochemical behaviour, another series of experiments was performed after an interval of 10 passages from the preceding three experiments revealing the same results (not shown). After trypsinization and harvesting, cells were transferred into 6-well plates (TPP Techno Plastic Products AG, Buchs, CH) at a concentration of 2 × 10^5 cells per well. The subsequent in vitro decidualization protocol was as described in Kautz et al. (2015). In short, following seeding, cells were pre-incubated for 24 h, and then, after washing with PBS the serum-containing medium was replaced by stimulation medium containing 0.1% bovine serum albumin (BSA) instead of FBS, and the decidualization protocol was applied for 72 h. For this, cells were treated with 0.5 mM N6,2′-O-dibutyryladenosine-3′,5′-cyclic monophosphate (dbcAMP); non-treated cells served as control. Additionally, cells were stimulated over 72 h with 0.1 µM, 1 µM and 10 µM PGE2 (P0409; Sigma-Aldrich Chemie GmbH, Buchs, CH).

For immunocytochemistry, sterile glass cover slips were placed into the wells to allow the seeded cells to adhere to them. The immunofluorescence staining procedure was as described previously (Kowalewski et al., 2013). The following primary antibodies were used: monoclonal mouse anti-VIMENTIN, an intermediate filament protein and marker of mesenchymal type cells, such as fibroblast and endothelial cells (same as for IHC), dilution 1:100; affinity-purified polyclonal rabbit anti-CYTOKERATIN, wide-spectrum screening, dilution 1:300 (both from Dako Schweiz AG); monoclonal mouse anti-SV40T-antigen (dilution 1:200; ab16879, Abcam, Cambridge, UK); and monoclonal rabbit anti-VIMENTIN (dilution 1:500, ab92547, Abcam). Secondary antibodies were applied in combination with nuclear 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), which was used at a concentration of 1:1000. The secondary antibodies used were as

follows: goat anti-rabbit IgG antibody FI-1000 and goat anti-mouse FI-2000 antibody, both from Vector Laboratories Ltd, both at 1:200 dilution. As controls, staining with only primary or secondary antibodies was performed, while autofluorescence was checked by omitting any antibodies. The target specificity of VIMENTIN and CYTOKERATIN was proved in our previous reports (Kautz et al., 2015; Kowalewski et al., 2010).

2.4 | Western blot detection of SV40T-antigen protein expression in immortalized canine uterine stromal cells

For Western blot analysis, cells were collected from 6-well plates using NET-2 lysis buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.05% NP-40) containing 10 µl/ml protease inhibitor (P8340; Sigma-Aldrich). A detailed protocol can be found in Gram et al. (2013). In short, after separation using a 10% polyacrylamide gel, immunoblotting was performed on polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories GmbH, Munich, DE), blocked with 5% low-fat milk powder in 0.25% Tween-20 in PBS (PBST) solution. The primary antibody was monoclonal mouse anti-SV40T-antigen (dilution 1:500, ab16879; Abcam). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (W402B; Promega, Dübendorf, CH) at a dilution of 1:15000. Signals were detected in a ChemiDoc XRS+ System with Image Lab Software from Bio-Rad, using the SuperSignal West chemiluminescent substrate (Life Technologies Europe B.V., Zug, CH). After detection of the SV40Tag protein, the membrane was reblotted with monoclonal mouse anti-β-actin antibody for a loading control (1:1000, sc-69879, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

2.5 | RNA isolation, reverse transcription, semi-quantitative (TaqMan) PCR and evaluation of data

Total RNA was isolated using TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNase treatment was performed following the supplier's protocol using RQ1 RNase-free DNase (Promega). Afterwards, complementary DNA (cDNA) synthesis was performed using RT reagents from Applied Biosystems, Foster City, CA, USA. A detailed protocol for the preparations and for the semi-quantitative real-time (TaqMan) PCR has been published previously (Kautz et al., 2015; Kowalewski, Meyer, Hoffmann, Aslan, & Boos, 2011; Kowalewski et al., 2006). TaqMan PCR was run with Fast Start Universal Probe Master (ROX®) (Roche Diagnostics AG, CH) using cDNA corresponding to 200 ng of DNase-treated total RNA per sample. All reactions were run in duplicate in an automated fluorometer (ABI PRISM® 7500 Sequence Detection System, Applied Biosystems). For negative controls, autoclaved water instead of cDNA and the RT-minus control were used (Kowalewski et al., 2006, 2011). The results were normalized against three reference genes (GAPDH, *cyclophilin A* and *actinB*). The list of primers and 6-FAM- and TAMRA-labelled (TaqMan) probes purchased from Microsynth, Balgach, CH, is presented in Table 1. Before use, their efficiency was established as previously described (Kowalewski et al., 2011). The following canine-specific TaqMan Gene Expression Assays were ordered from Applied Biosystems: *cyclophilin A* (Prod. No. Cf03986523-gH), *IGF1* (Prod. No. Cf02627846_m1), *IGF2*

TABLE 1 List of primers and TaqMan probes used for semi-quantitative RT-PCR

Primer	Accession numbers	Primer sequence		Product length (bp)
GAPDH	AB028142	Forward	5′-GCT GCC AAA TAT GAC GAC ATC A-3′	75
		Reverse	5′-GTA GCC CAG GAT GCC TTT GAG-3′	
		TaqMan probe	5′-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3′	
PRLR	HQ267784	Forward	5′-GGATCT TTG CCG TTCTTT-3′	92
		Reverse	5′-AAG GAT GCA GGT CAC CAT GCT AT-3′	
		TaqMan probe	5′-ATT ATG GTC GTA GCA GTG GCT TTG AAA GGC-3′	
PGR	NM_001003074	Forward	5′-CGA GTC ATT ACC TCA GAA GAT TTG 1 1 1-3′	113
		Reverse	5′-CTT CCA TTG CCC TTTTAA AGA AG A-3′	
		TaqMan probe	5′-AAG CAT CAG GCT GTC ATT ATG GTG TCC TAA CTT-3′	
PTGES	NM_001122854	Forward	5′-GTC CTG GCG CTG GTG AGT-3′	89
		Reverse	5′-ATG ACA GCC ACC ACG TAC ATC-3′	
		TaqMan probe	5′-TCC CAG CCTTCC TGC TCT GCA GC-3′	
PTGER2/EP2	AF075602	Forward	5′-CAC CCT GCT GCT GCT TCT C-3′	78
		Reverse	5′-CGG TGC ATG CGG ATG AG-3′	
		TaqMan probe	5′-TGC TCG CCT GCA ACTTTC AGC GTC-3′	
PTGER4/EP4	NM_001003054	Forward	5′-AAA TCA GCA AAA ACC CAG ACT TG-3′	96
		Reverse	5′-GCA CGG TCT TCC GCA GAA-3′	
		TaqMan probe	5′-ATC CGA ATT GCT GCT GTG AAC CCT ATC C-3′	

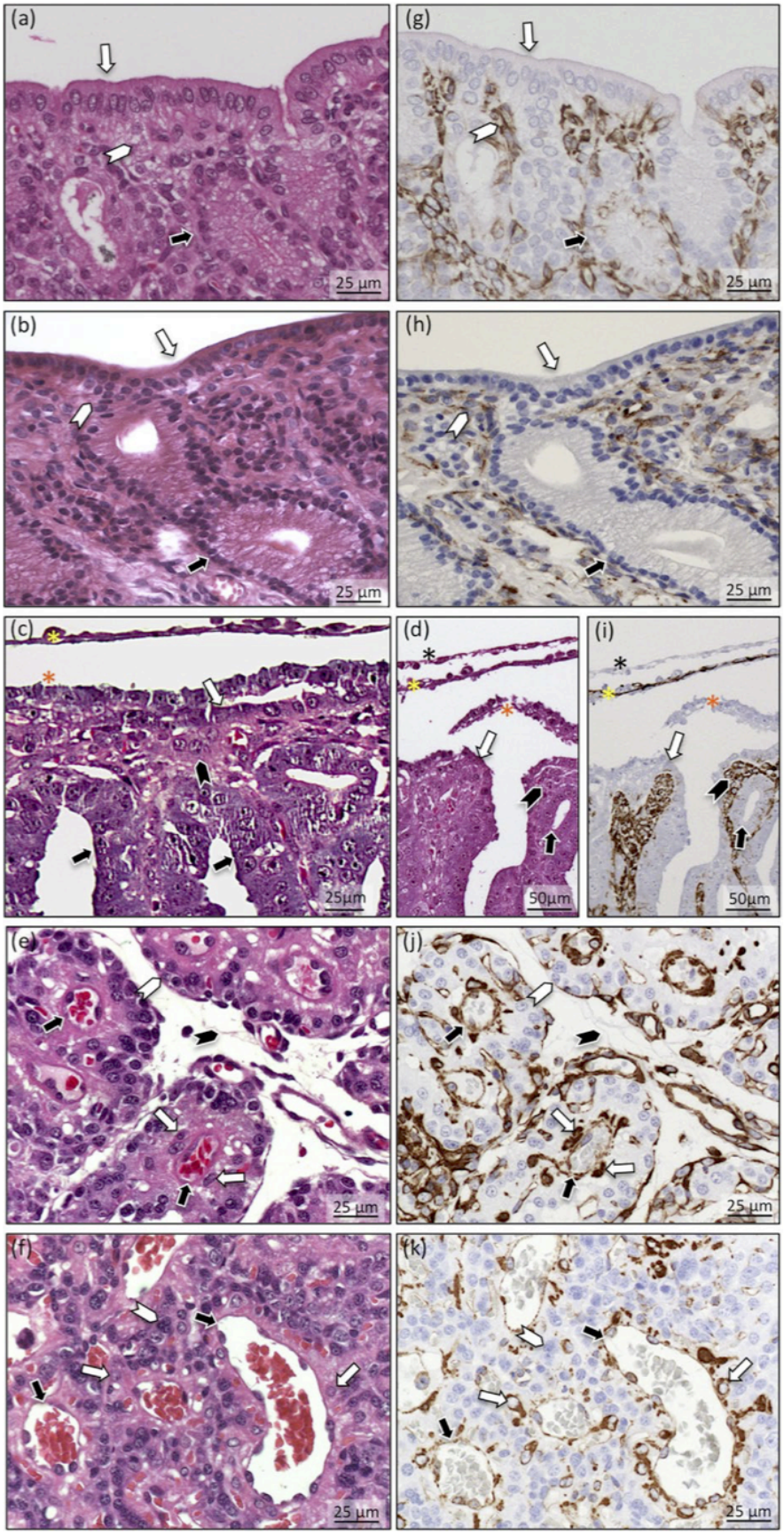


FIGURE 1 Morphological changes associated with the process of canine decidualization in vivo. Haematoxylin and eosin staining (HE; a–f) and immunohistochemical detection of VIMENTIN (g–k) are shown. (a, g) Non-pregnant canine uterus at early dioestrus; (b, h) early-pregnant (pre-implantation) canine uterus; (c, d, i) canine uterus at the implantation stage, HE staining is shown at higher and lower magnification (day 17 of embryonal life); (e, j) midgestation placenta; (f, k) placenta at pre-partum luteolysis. Prior to placentation (a–d, g–i): open arrows = surface (luminal) epithelium; solid arrows = glandular epithelium; open arrowheads = subepithelial compartments prior to morphological decidualization; solid arrowheads = subepithelial decidualization at the time of implantation. In c, d and i: black asterisk = endoderm; yellow asterisk = mesoderm; orange asterisk = trophoblast. Following placentation (e, f, j, k): open arrows = decidual cells; solid arrows = maternal blood vessels; open arrowheads = foetal trophoblast (cytotrophoblast is indicated); solid arrowheads = foetal stromal compartment

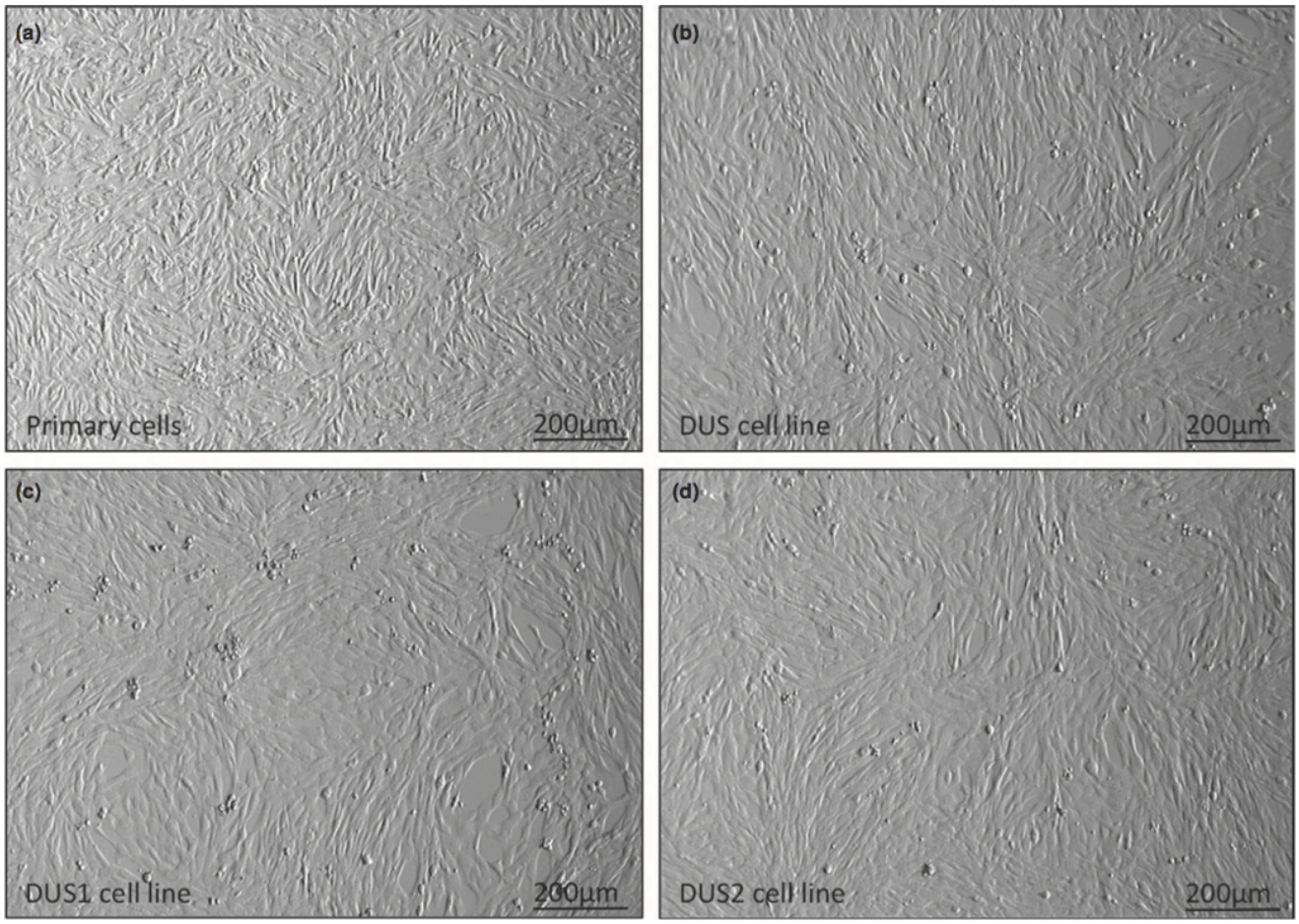


FIGURE 2 Morphological appearance of primary canine uterine stromal cells and immortalized cell lines at high confluence. a) Primary canine uterine stromal cells; b) cell line DUS at $p = 7$; c) cell line DUS1 at $p = 8$; d) cell line DUS2 at $p = 10$

(Prod. No. Cf02647136_m1) and *actinB* (Prod. No. Cf03023880_g1). For relative quantification, the comparative CT method (ΔΔCT method) was applied as previously described (Kowalewski et al., 2010, 2011).

An unpaired, two-tailed Student's *t* test was performed in experiments assessing the cAMP-driven decidualization of DUS cells, and one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple-comparison test was applied for the PGE2-experiments. Numerical data are presented as mean ± *SD*. GraphPad 3.06 software was used for all analyses (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Characterization of decidual cell development in the canine uterus and placenta in vivo

Morphological changes associated with the process of canine stromal cell decidualization were observed in vivo. Consecutive histological sections of canine uterine or utero-placental samples were stained with HE (Figure 1a–f) and by IHC detecting VIMENTIN (Figure 1g–k); this procedure facilitates differentiation of canine decidual cells (Kowalewski et al., 2011). The morphological appearance of stromal compartments did not differ strongly between the first two groups investigated, that is non-pregnant and early-pregnant canine uteri (Figure 1a,b). Typical stromal VIMENTIN staining was noted throughout all mesenchymal compartments (Figure 1g,h). Commencing with the more intimate contact between maternal and foetal tissues during the implantation period (group 3), strong endometrial proliferation was associated with histomorphological changes in the subepithelial stromal compartments (Figure 1c,d,i). This tissue remodelling included swelling of VIMENTIN-positive cells, which became larger and rounded, implying an ongoing decidualization process (Figure 1c,d,i). In group 4 (midgestation), clearly distinguishable VIMENTIN-positive canine decidual cells could be found in close proximity to maternal blood vessels (Figure 1e,j). The mature, preterm placenta is represented by group 5 (luteolysis) (Figure 1f,k).

3.2 | Establishment of stably transfected, immortalized dog uterine stromal cell lines

Canine uterine stromal cells were isolated using our previously established protocol (Kautz et al., 2015) (Figure 2). Immortalization was achieved by a stable transfection of cells at $p = 0$ with the oncogene

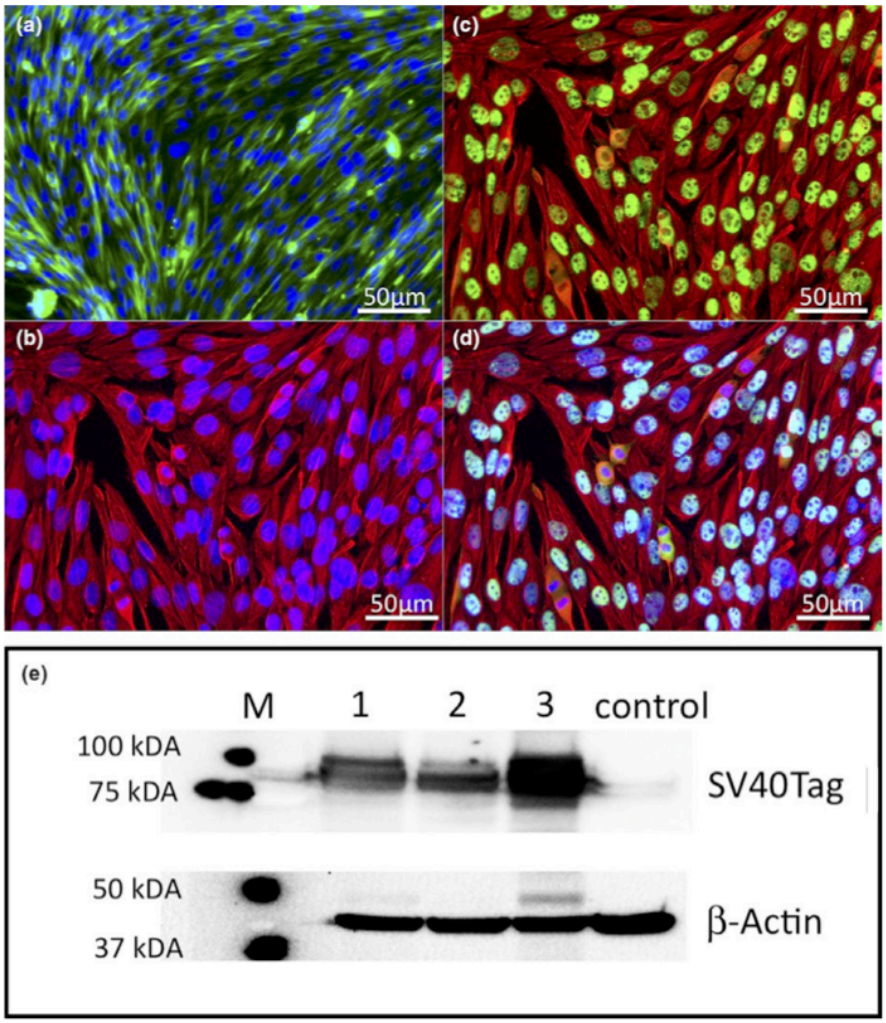


FIGURE 3 Immortalized canine uterine stromal cell line (DUS). (a) VIMENTIN (green) and CYTOKERATIN (red) staining was performed to verify the mesenchymal character of DUS cell line. Cells are devoid of any CYTOKERATIN signals indicating they are of high purity and retained stromal characteristics. Nuclear staining was achieved with 4′,6-diamidino-2-phenylindole (DAPI); (b–c) triple staining with VIMENTIN (red), SV40Tag protein (green nuclear staining) and DAPI (blue). Shown are (b) VIMENTIN and DAPI and (c) VIMENTIN and SV40Tag. (d) Shown is a merged image of (b) and (c). Western blot detection of SV40Tag protein (approximately 80 kDa; *M* = molecular weight marker) in DUS, DUS1 and DUS2 cell lines (1–3, respectively) is shown in (e). Cells were collected and homogenized, and 20 µg of the lysate was used for Western blots. Primary, non-immortalized canine uterine stromal cells were used for a negative control. β-Actin was used for the loading control

pSV40Tag-expressing vector. Three monolayer cell lines were generated: DUS, DUS1 and DUS2. Following transfection, cells retained their spindle-shaped morphology throughout the experiments (Figure 2). Each cell line went through at least 25–30 passages without changing its morphological characteristics and without any signs of senescence or growth retardation.

For each experiment, immunofluorescence staining was performed and the mesenchymal origin of immortalized cells was verified by nearly 100% positive signals for VIMENTIN (Figure 3b) in double staining with wide-spectrum CYTOKERATIN (Figure 3a). The cells exhibited stable expression of SV40Tag protein in their nuclei (Figure 3c,d). The clearly detectable presence of SV40Tag protein was confirmed by immunoblotting, with all three cell lines displaying a strong signal at approximately 80 kDA, while non-transfected cells generated only weak background staining (Figure 3e).

3.3 | In vitro decidualization of immortalized canine uterine stromal cell lines

All three newly established cell lines (DUS, DUS1 and DUS2) were submitted to our previously described dbcAMP-mediated in vitro decidualization protocol, and the gene expression of selected decidualization markers (*PRLR*, *IGF1*, *IGF2*, *PGR*, *PTGES*, *PTGER2/EP2*, *PTGER4/EP4*) was monitored following 72 h stimulation with 0.5 mM dbcAMP (Kautz et al., 2015). All genes were clearly detectable in both stimulated and unstimulated cells. Their morphology during the stimulation period indicated an ongoing decidualization process; however, cell lines DUS1 and DUS2 failed to respond to dbcAMP treatment and revealed unchanged levels of transcripts encoding for *PRLR* (DUS1), *PGR* (DUS1 and DUS2) and *PTGES* (DUS2) ($p > 0.05$, not shown). The cell line designated as DUS revealed the widest expression spectrum of positively affected decidualization markers in response to dbcAMP compared with its non-treated counterparts. The expression levels are presented in Figure 4. Thus, the mRNA encoding for *PRLR*, *IGF1* and *EP4* increased significantly with $p = 0.0001$, *PGR* was upregulated with $p = 0.03$, *PTGES* was strongly elevated with $p = 0.0007$ and *EP2* increased with $p = 0.001$ (Figure 4), compared with the respective controls. Yet, the expression of *IGF2* mRNA remained unaffected ($p > 0.05$). Next, the decidualization potential of PGE2 was tested in the DUS cell line during a 72 h time course (Figure 5). Whereas the expression of *IGF1*, *IGF2* and *EP2* did not change significantly in response to treatment with PGE2 ($p > 0.05$), the expression of

PRLR mRNA increased in response to 1.0 µM PGE2 ($p < 0.05$) and was further potentiated ($p < 0.01$) by responding positively when 10.0 µM PGE2 was used (Figure 5). Similarly, the expression of *PGR* mRNA increased significantly in response to stimulation with 1.0 µM and 10.0 µM PGE2 ($p < 0.01$ and $p < 0.05$, respectively). *EP4* mRNA responded positively with $p < 0.05$ to both 1.0 and 10.0 µM PGE2 (Figure 5). *PTGES* was altered in stimulated cells, which exhibited elevated mRNA levels in response to 10.0 µM PGE2 ($p < 0.05$).

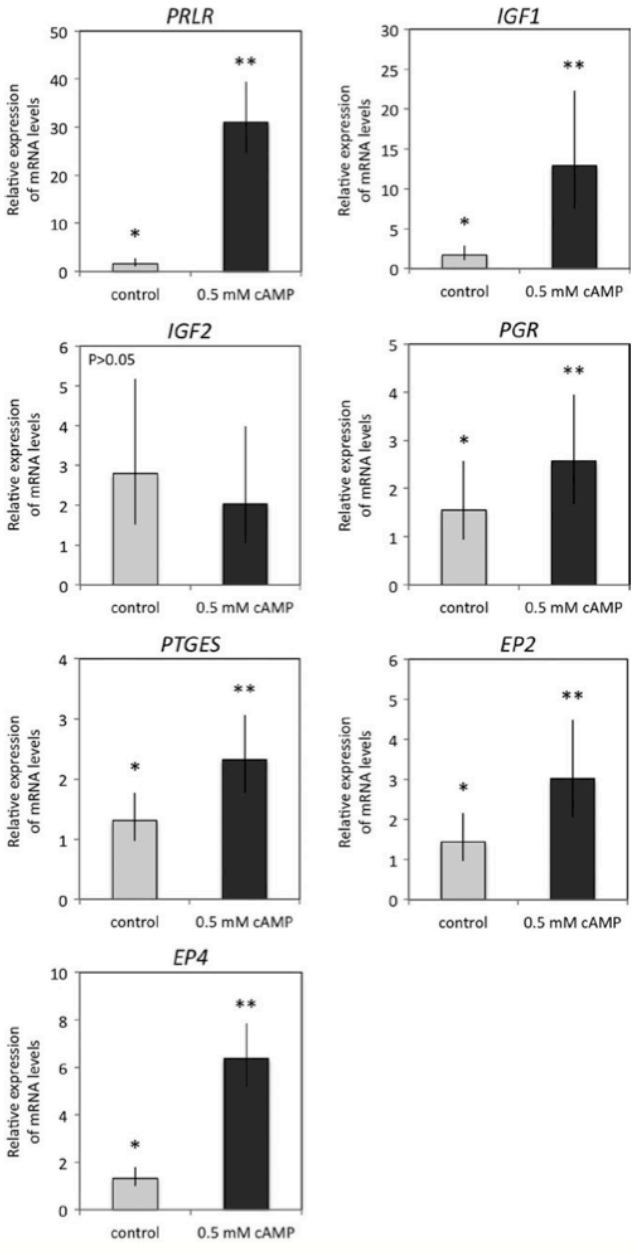


FIGURE 4 Expression of prolactin receptor (*PRLR*), insulin-like growth factor (*IGF*) 1 and *IGF2*, progesterone receptor (*PGR*), PGE2 synthase (*PTGES*) and PGE2 receptors 2 (*PTGER2/EP2*) and 4 (*PTGER4/EP4*), as determined by real-time (TaqMan) PCR in the DUS cell line. Cells were treated with 0.5 mM dbcAMP for 72 h. An unpaired, two-tailed Student's *t* test was applied. Bars with different asterisks differ at: $p = 0.0001$ (*PRLR*, *IGF1* and *EP4*), $p = 0.001$ (*EP2*), $p = 0.03$ (*PGR*), $p = 0.0007$ (*PTGES*). Numerical data are presented as mean ± standard deviation (*SD*)

4 | DISCUSSION

Decidualization involves morphological and functional reprogramming of maternal stromal cells during their transition into decidual cells. In species exhibiting this process, failure to decidualize results in pregnancy loss. Even though not as strongly pronounced as in rodents or humans, decidualization can be also observed in the canine uterus. Being capable of transmitting signals to the uterus, early preimplantation canine embryos invoke modulatory effects on genes known for their involvement in the processes of implantation and placentation (Kautz et al., 2014). The changes associated with this early decidualization process seem, however, to be predominantly functional and biochemical, and less of a structural nature. Starting with the attachment of trophoblast to the maternal uterine surface, the embryo-driven decidualization process becomes more apparent. This has been strikingly shown in the present study by observing morphological changes of interstitial stromal cells in the luminal subepithelial compartments, which can be clearly interpreted as the onset of canine morphofunctional decidualization. This process develops further towards midgestation, resulting in the formation of morphologically clearly distinguishable decidual cells. In the fully developed canine placenta, these cells can be found predominantly in close proximity to large maternal vessels. This may indicate that, at least in part, decidual cells develop from the *tunica media* of those vessels, sharing with them the expression of both VIMENTIN and smooth muscle α-actin (αSMA) (Kautz et al., 2015). This hypothesis needs, however, to be verified.

Driven by the critical importance of decidual cells for the maintenance of canine pregnancy (Gram et al., 2014; Kowalewski et al., 2010), in our previous study aimed at understanding the basic mechanisms of canine decidualization, we have developed an in vitro model of canine decidualization (Kautz et al., 2015). Here, in addition to very limited access to fresh tissue material, our goal was to establish immortalized canine stromal cell lines, allowing us to avoid the senescence of primary cells that are cultured for prolonged passages.

Cells isolated from naturally oestrogenized early diestric bitches were successfully and stably transfected with a vector expressing the large T-antigen of simian vacuolating virus 40 (pSV40Tag). Acting on many steps related to cell transformation, T-antigen, among others affects key proteins involved in controlling cell growth. In particular it inhibits the tumour suppressor proteins p53, pRb, p107 and p130/pRb2, promoting transformation and immortalization of cells (Butel & Lednicky, 1999; Kim, Banga, Jha, & Ozer, 1998). The stable expression of SV40Tag protein in three newly established cell lines was verified both by cytochemical staining and immunoblotting. The immortalized cells retained their biochemical and morphological characteristics for over 30 passages, continuously responding positively to VIMENTIN staining. This is in extreme contrast to primary canine uterine stromal cells that typically undergo senescence after 2–3 passages and

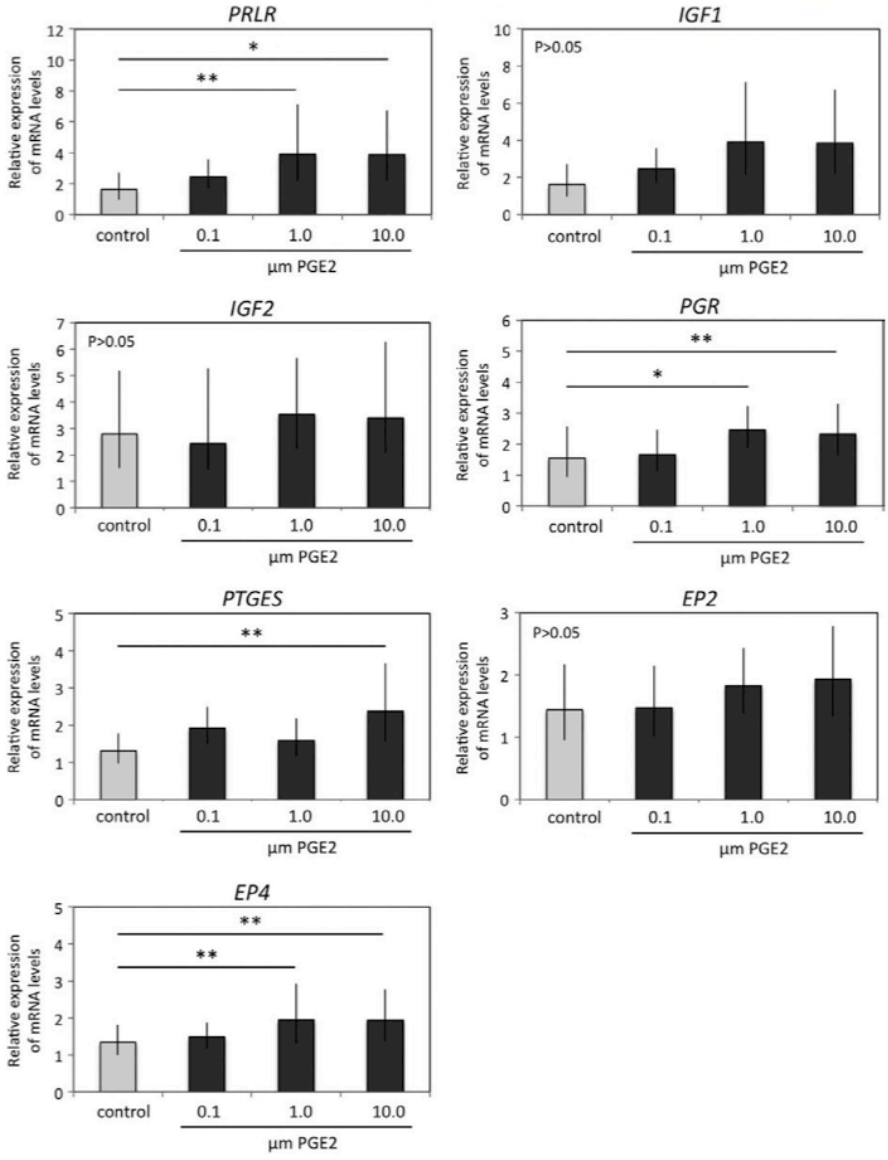


FIGURE 5 Insulin-like growth factor (*IGF*) 1 and *IGF2*, progesterone receptor (*PGR*), PGE2 synthase (*PTGES*) and PGE2-receptors *PTGER2/EP2* and *PTGER4/EP4*, as determined by real-time (TaqMan) PCR in DUS cells treated with increasing doses of PGE2 (0.0, 0.1, 1.0 and 10.0 µM). The treatment was applied for 72 h. One-way ANOVA was applied ($p = 0.007$ for *PRLR*; $p > 0.05$ for *IGF1*; $p > 0.05$ for *IGF2*; $p = 0.01$ for *PGR*; $p = 0.004$ for *PTGES*; $p > 0.05$ for *EP2*; and $p = 0.04$ for *EP4*) followed by the Tukey–Kramer multiple-comparison test; all samples were compared against the non-treated control in each group. Numerical data are presented as mean ± standard deviation (*SD*). Bars with different asterisks differ at: (*) $p < 0.01$, (**) $p < 0.05$

concomitantly display signs of functional and morphological differentiation, for example by expressing CYTOKERATIN.

The basic capability of immortalized cell lines to undergo in vitro decidualization was assessed by applying our dbcAMP-mediated decidualization protocol over a 72 h time course and investigating expression of some of the factors previously established as markers of canine decidualization (Kautz et al., 2014, 2015). Of the three immortalized cell lines, only one, designated as DUS, was found to express the widest spectrum of decidualization markers, resembling that observed previously with primary stromal cells and during early in vivo decidualization (Kautz et al., 2014, 2015). This concerns predominantly genes such as *PRLR*, *IGF1* and *PTGES*, the expression of which was strongly upregulated in decidualized DUS cells. The expression of *IGF2*, which was upregulated in primary uterine stromal cells (Kautz et al., 2015), remained unaffected by 0.5 mM dbcAMP in the DUS cell line. However, whereas in that previous study *IGF1* was revealed to be a fast responder by showing a significant increase within 24 h towards dbcAMP stimulation, the response of *IGF2* was slower and increased only at the end of the stimulation time course while displaying high variation (Kautz et al., 2015). It thus seems plausible that the deviating expression patterns of *IGF2* mRNA expression observed between our two studies may be related to experimental conditions and/or individual variations. Also contrasting with primary cells, but matching the profile of in vivo decidualization, the newly established DUS cell line additionally showed increased expression of *PGR*, *PTGER2/EP2* and *PTGER4/EP4*. However, as the present study was conducted only at the messenger level, no further conclusions can be drawn until the expression of the respective proteins can be investigated.

The potential role of PGE2 during canine decidualization has been investigated for the first time in vitro, adding a new functional aspect to studies on this process. In accordance with the increased expression of *PTGES* during canine decidualization in vivo and in vitro (as also confirmed in this study), we were able to show that PGE2 exerts positive stimulatory effects on expression of *PRLR* and *PGR*. This is an

interesting finding, as the P4-dependent and cAMP-mediated decidualization has been shown to be accelerated by PGE2 in, for example, human endometrial stromal cells (Brar et al., 1997). Keeping in mind that decidual cells are the only cellular constituents expressing PGR within the canine placenta, it seems plausible that similar effects could apply for the dog. Therefore, our in vitro model suggests an indirect functional involvement of PGE2 in the P4-dependent establishment and maintenance of canine gestation due to its modulatory effects on PGR expression. Finally, PGE2 seems also to be involved in a positive feedback loop by upregulating the expression of its own synthase (*PTGES*) and one of its G-protein-coupled, cAMP/PKA-mediating receptors, namely *PTGER4/EP4*.

In conclusion, the process of decidualization is indispensable for successful canine pregnancy and needs, therefore, to be thoroughly investigated. In addition to a closer morphological characterization of canine in vivo decidualization, beginning with the pre-implantation stage and up to the fully mature pre-partum placenta, our study provides a valuable in vitro model using an immortalized DUS cell line. This will allow future, more detailed studies of the underlying mechanisms governing the process of canine decidualization. The basic functionality of the newly established cell line has been validated in protocols involving dbcAMP- and PGE2-induced decidualization. Finally, PGE2 has been introduced as a new, potentially important factor involved in P4-dependent and PGR-mediated decidualization in the dog.

ACKNOWLEDGEMENTS

The technical expertise and contribution of Elisabeth Högger is gratefully appreciated. The authors are grateful to Prof. Bernd Hoffmann, Justus-Liebig University, Giessen, Germany, and Prof. Selim Aslan, Near East University, Nicosia, Cyprus, and to the "Hospital Veterinário do Baixo Vouga," Estrada Nacional 1, 355 - Segadães, 3750-742, Águeda, Portugal, for the kind provision of the tissue material, and to Dr. Barry Bavister for careful editing of the manuscript. Part of the laboratory work was performed using the logistics at the Center for Clinical Studies, Vetsuisse Faculty, University of Zurich.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. All authors read and approved the final version of the manuscript.

REFERENCES

Bazer, F. W., Thatcher, W. W., Hansen, P. J., Mirando, M. A., Ott, T. L., & Plante, C. (1991). Physiological mechanisms of pregnancy recognition in ruminants. *Journal of Reproduction and Fertility*. Supplement, *43*, 39–47.

Brar, A. K., Frank, G. R., Kessler, C. A., Cedars, M. I., & Handwerger, S. (1997). Progesterone-dependent decidualization of the human endometrium is mediated by cAMP. *Endocrine*, *6*, 301–307.

Butel, J. S., & Lednicky, J. A. (1999). Cell and molecular biology of simian virus 40: Implications for human infections and disease. *Journal of the National Cancer Institute*, *91*, 119–134.

Chrusciel, M., Bodek, G., Kirtiklis, L., Lewczuk, B., Hyder, C. L., Blitek, A., … Andronowska, A. (2011). Immortalization of swine umbilical vein endothelial cells (SUVECs) with the simian virus 40 large-T antigen. *Molecular Reproduction and Development*, *78*, 597–610.

Concannon, P. W., McCann, J. P., & Temple, M. (1989). Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *Journal of Reproduction and Fertility*. Supplement, *39*, 3–25.

Gellersen, B., & Brosens, J. (2003). Cyclic AMP and progesterone receptor cross-talk in human endometrium: A decidualizing affair. *The Journal of Endocrinology*, *178*, 357–372.

Gram, A., Buchler, U., Boos, A., Hoffmann, B., & Kowalewski, M. P. (2013). Biosynthesis and degradation of canine placental prostaglandins: Prepartum changes in expression and function of prostaglandin F2alpha-synthase (PGFS, AKR1C3) and 15-hydroxyprostaglandin dehydrogenase (HPGD). *Biology of Reproduction*, *89*, 2.

Gram, A., Fox, B., Buchler, U., Boos, A., Hoffmann, B., & Kowalewski, M. P. (2014). Canine placental prostaglandin E2 synthase: Expression, localization, and biological functions in providing substrates for prepartum PGF2alpha synthesis. *Biology of Reproduction*, *91*, 154.

Gram, A., Hoffmann, B., Boos, A., & Kowalewski, M. P. (2015). Expression and localization of vascular endothelial growth factor A (VEGFA) and its two receptors (VEGFR1/FLT1 and VEGFR2/FLK1/KDR) in the canine corpus luteum and utero-placental compartments during pregnancy and at normal and induced parturition. *General and Comparative Endocrinology*, *223*, 54–65.

Hoffmann, B., Hoveler, R., Hasan, S. H., & Failing, K. (1992). Ovarian and pituitary function in dogs after hysterectomy. *Journal of Reproduction and Fertility*, *96*, 837–845.

Kautz, E., de Carvalho Papa, P., Reichler, I. M., Gram, A., Boos, A., & Kowalewski, M. P. (2015). In vitro decidualisation of canine uterine stromal cells. *Reproductive Biology and Endocrinology: RB&E*, *13*, 85.

Kautz, E., Gram, A., Aslan, S., Ay, S. S., Selcuk, M., Kanca, H., … Kowalewski, M. P. (2014). Expression of genes involved in the embryo-maternal interaction in the early-pregnant canine uterus. *Reproduction*, *147*, 703–717.

Kennedy, T. G., & Doktorcik, P. E. (1988). Effects of analogues of prostaglandin E2 and F2 alpha on the decidual cell reaction in the rat. *Prostaglandins*, *35*, 207–219.

Kim, S. H., Banga, S., Jha, K. K., & Ozer, H. L. (1998). SV40-mediated transformation and immortalization of human cells. *Developments in Biological Standardization*, *94*, 297–302.

Korzekwa, A. J., Bodek, G., Bukowska, J., Blitek, A., & Skarzynski, D. J. (2011). Characterization of bovine immortalized luteal endothelial cells: Action of cytokines on production and content of arachidonic acid metabolites. *Reproductive Biology and Endocrinology: RB&E*, *9*, 27.

Kowalewski, M. P., Beceriklisoy, H. B., Aslan, S., Agaoglu, A. R., & Hoffmann, B. (2009). Time related changes in luteal prostaglandin synthesis and steroidogenic capacity during pregnancy, normal and antiprogestin induced luteolysis in the bitch. *Animal Reproduction Science*, *116*, 129–138.

Kowalewski, M. P., Beceriklisoy, H. B., Pfarrer, C., Aslan, S., Kindahl, H., Kucukaslan, I., & Hoffmann, B. (2010). Canine placenta: A source of prepartal prostaglandins during normal and antiprogestin-induced parturition. *Reproduction*, *139*, 655–664.

Kowalewski, M. P., Fox, B., Gram, A., Boos, A., & Reichler, I. (2013). Prostaglandin E2 functions as a luteotrophic factor in the dog. *Reproduction*, *145*, 213–226.

Kowalewski, M. P., Meyer, A., Hoffmann, B., Aslan, S., & Boos, A. (2011). Expression and functional implications of peroxisome proliferator-activated receptor gamma (PPARgamma) in canine reproductive tissues during normal pregnancy and parturition and at antiprogestin induced abortion. *Theriogenology*, *75*, 877–886.

Kowalewski, M. P., Mutembei, H. M., & Hoffmann, B. (2008). Canine prostaglandin E2 synthase (PGES) and its receptors (EP2 and EP4): Expression in the corpus luteum during dioestrus. *Animal Reproduction Science*, *109*, 319–329.

Kowalewski, M. P., Schuler, G., Taubert, A., Engel, E., & Hoffmann, B. (2006). Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. *Theriogenology*, *66*, 1423–1430.

Parhar, R. S., Yagel, S., & Lala, P. K. (1989). PGE2-mediated immunosuppression by first trimester human decidual cells blocks activation of maternal leukocytes in the decidua with potential anti-trophoblast activity. *Cellular Immunology*, *120*, 61–74.

Perry, J. S., Heap, R. B., & Amoroso, E. C. (1973). Steroid hormone production by pig blastocysts. *Nature*, *245*, 45–47.

Schäfer-Somi, S., Sabitzer, S., Klein, D., Reinbacher, E., Kanca, H., Beceriklisoy, H. B., … Aslan, S. (2013). Vascular endothelial (VEGF) and epithelial growth factor (EGF) as well as platelet-activating factor (PAF) and receptors are expressed in the early pregnant canine uterus. *Reproduction in Domestic Animals*, *48*, 20–26.

Spencer, T. E., & Bazer, F. W. (2004). Conceptus signals for establishment and maintenance of pregnancy. *Reproductive Biology and Endocrinology: RB&E*, *2*, 49.

Steinetz, B. G., Goldsmith, L. T., Harvey, H. J., & Lust, G. (1989). Serum relaxin and progesterone concentrations in pregnant, pseudopregnant, and ovariectomized, progestin-treated pregnant bitches: Detection of relaxin as a marker of pregnancy. *American Journal of Veterinary Research*, *50*, 68–71.

Tamura, I., Asada, H., Maekawa, R., Tanabe, M., Lee, L., Taketani, T., … Sugino, N. (2012). Induction of IGFBP-1 expression by cAMP is associated with histone acetylation status of the promoter region in human endometrial stromal cells. *Endocrinology*, *153*, 5612–5621.

Vermeirsch, H., Simoens, P., & Lauwers, H. (2000). Immunohistochemical detection of the estrogen receptor-alpha and progesterone receptor in the canine pregnant uterus and placental labyrinth. *The Anatomical Record*, *260*, 42–50.

Yee, G. M., & Kennedy, T. G. (1993). Prostaglandin E2, cAMP and cAMP-dependent protein kinase isozymes during decidualization of rat endometrial stromal cells in vitro. *Prostaglandins*, *46*, 117–138.

Discussion

Embryo-maternal crosstalk and its effects on uterine ECM in the dog

Species-specific embryo-maternal crosstalk at the beginning of canine gestation was indicated by previous studies, including those from our laboratory, which identified differential uterine gene expression between non-pregnant and early pregnant bitches (pre-implantation/pre-attachment stage of pregnancy) [19, 20, 26, 27, 29, 90]. These studies focused predominantly on uterine expression of particular genes that reflected changes in single molecular systems. Thus, a comprehensive approach was missing, that would allow detection of global embryo-induced uterine changes that are presumably mandatory for successful establishment of canine pregnancy. This became possible by applying modern molecular tools such as microarray analysis, allowing us to perform more detailed, in-depth studies.

Therefore, we investigated the uterine response to the presence of free-floating embryos (i.e., prior to the attachment of blastocysts to the uterus) in the uterine lumen through DNA microarray analysis. A total of 15,668 genes was detected [101]. A mandatory step in analyzing microarray results was to adjust the data for multiple testing, therefore we applied a false discovery rate (FDR) of 10% (i.e., adjusted P-value <0.1) [101]. The P-value (P) was set at P<0.01 [101]. This narrowed the total gene set down to 412 differentially expressed genes (DEG). Of these, 314 genes were more highly and 98 genes were less expressed in the early pregnant group [101]. The three genes with the highest individual fold-enrichment in response to the presence of free-floating embryos were: importin 9 (IPO9), inter-alpha-trypsin inhibitor heavy chain family member 4 (ITIH4) and nephroblastoma overexpressed (NOV) [101]. These genes are involved in cell differentiation, proliferation and matrix organization, biological processes which seem to reflect the first pregnancy-associated changes in the uterine tissue that are initiated before the attachment of the blastocyst to the uterine lumen. The three least expressed genes (i.e., genes whose expression was most strongly suppressed in the early pregnant uterus) were related to cellular proliferation (Ki-67/MKI67), maintenance of cellular cytoskeleton (diaphanous related formin 3 (DIAPH3)), and regulation of the bioavailability of local IGFs (pappalysin (PAPPA) 2) [101]. With regards to PAPPA2, in the dog, its possible functions in regulating local IGFs, and its potential involvement in decidualization, implantation and trophoblast invasion, still need to be investigated in more detail. Nevertheless, our microarray data indicate that PAPPA2 is a worthy target for future studies on canine decidualization and pregnancy.

In order to identify global embryo-induced uterine changes, the 412 detected DEG (FDR 10%, P<0.01) were analyzed for the presence of over- and under-represented functional terms (gene ontologies), networks and pathways. The response of the canine uterus to the presence of free-floating blastocysts turned out to be associated with an enhanced presence of terms referring to ECM region, inflammatory response, regulation of cell motion and cell migration and angiogenesis [101]. The downregulated genes were predominantly associated with focal adhesion, cell morphogenesis, cellular component morphogenesis, cell adhesion and

fibronectin type 3 [101]. These findings were confirmed by applying two independent software tools (i.e., IPA and DAVID) utilizing independent data bases and algorithms, emphasizing the reliability of the presented results [101]. Additionally, a comprehensive list of candidate genes (i.e., allograft inflammatory factor (AIF) 1, chemokine ligand (CXCL) 16, chemokine receptor (CXCR) 6 and -7, liver X receptor (LXR), prostaglandin D2 receptor (PTGDR), laminin alpha (LAMA) 2, idolamin 2,3-dioxygenase (IDO) 1, tissue inhibitor of matrix metalloproteinase (TIMP) 2 and PAPPA2) was used to verify the results of microarray analysis [101].

Clearly, the most strongly upregulated molecular system found in early canine pregnancy (compared to non-pregnant controls), was the "ECM region" presenting an enrichment score of 10.77, in which 185 genes were significantly overrepresented [101].
Consequently, because, in the literature available so far modulation of the uterine ECM during canine pregnancy seemed overlooked, we felt prompted to investigate the spatio-temporal expression of selected uterine ECM proteins at defined stages of canine pregnancy [102]. Our microarray data served as the basis for target selection. Utilizing semi-quantitative qPCR, mRNA expression was assessed, while immunohistochemistry (IHC) was performed to investigate the localization of ECM proteins [102].
The investigated ECM proteins were grouped functionally in proteins involved in cell-to-cell communication (e.g., connexin (Cx) 26 and -43), proteins involved in inhibition of matrix metalloproteinases (e.g., tissue inhibitor of metalloproteinases (TIMPs) 2 and -4), proteins of the basal lamina (e.g., extracellular matrix protein 1 (ECM1) and laminin alpha (LAMA) 2), or in proteins with structural and adhesive functions (e.g., alpha smooth muscle actin (αSMA), fibronectin (FN) 1 and collagens (COL) 1, -3 and -4) [102]. We were able to show that the canine uterine ECM undergoes only moderate modifications in early (pre-attachment) pregnancy. Among the ECM related proteins that were modulated by the presence of embryos, we found an embryo-mediated decrease in FN1 mRNA levels, whereas ECM1, LAMA2, TIMP2 and TIMP4 were increased [101, 102]. This uterine biochemical response towards the presence of free-floating blastocysts in the uterine lumen seems to be related to the modulation of trophoblast invasion, as well as to the proliferative and adhesive functions of the uterus [102].
It needs to be emphasized that, although biochemical differentiation associated with ECM remodeling is observed in the early pregnant (pre-attachment) uterus, morphological changes are absent at this time [103]. Vigorous reorganization processes in the uterus are found later, commencing with implantation and placentation [102]. Accordingly, the first apparent morphological changes (morphological decidualization) occur at day 17 of pregnancy, triggered by trophoblast attachment to the surface uterine epithelium [103]. This is in contrast to decidua formation in humans, where high circulating P4 levels trigger decidualization even in the absence of an invading trophoblast (reviewed in [35]).
Following attachment of the blastocyst to the uterus and subsequent formation of the placenta, utero-placental compartments (the whole thickness of the uterine wall together with adjacent

placenta), and the inter-placental uterine sites (parts of the placenta not attached to the placenta) were separately investigated for spatio-temporal expression of ECM proteins [102]. The utero-placental mRNA expression of structural collagens, COL1 and -3, and LAMA2 decreased from pre-implantation to post-implantation stages of pregnancy [102]. This first decrease of structural uterine ECM components appears to reflect the process of softening or loosening of the tissue in preparation for trophoblast invasion [102]. Following this, a further decrease in the expression of mRNA encoding for COL1, -3 and LAMA2 was noted at placentation sites towards prepartum luteolysis. These proteins were clearly detectable and localized in the myometrium and in all maternal and fetal stromal compartments [102]. In particular, the localization of these proteins in the connective tissue layer separating superficial glands (so-called glandular chambers) from deeper localized parts of the uterus drew our attention. In healthy pregnancy, proteolytic trophoblast activity is stopped in this part of the uterus, the connective tissue layer seems therefore to function as a barrier. The physiological shedding of the placenta following delivery of newborns occurs in the utero-placental compartments luminal to this structure. Thus, the strong decrease in expression of structural components (COL1, -3, and LAMA2) at prepartum luteolysis in the utero-placental compartments has been interpreted as a sign of preparation for the release of fetal membranes [102]. In contrast, mRNA expression of major structural collagens (COL1, -3 and -4) increased at the inter-placentation sites from pre-implantation towards mid-gestation [102]. It has been concluded that the increased presence of basement membranes and structural components is associated with growth of the uterus, which is a physiological process associated with the progression of pregnancy and development of conceptuses.

In addition to structural changes in the uterus and utero-placental compartments during pregnancy, we were interested in investigating intercellular communication processes promoted by gap junction molecules. The uterine expression of *macula communicans* structures (Connexins; Cx) has been observed before, e.g., in rat, human and sheep uteri throughout pregnancy [104-106]. In reproductive research, the spatio-temporal expression of Cx26 and -43 has been intensively studied. Interestingly, healthy pregnancy in rodents requires a complete suppression of both connexins prior to implantation [105, 107]. At implantation, connexins are re-induced in both spatial and temporal patterns [108]. In our study on canine uteri we observed that both Cx26 and Cx43 were not modulated by the presence of embryos prior to trophoblast attachment. Following implantation, the expression of Cx26 was initially strongly upregulated from pre-implantation towards post-implantation, however it decreased gradually towards prepartum luteolysis [102]. The expression of Cx43 decreased from pre-implantation to post-implantation, which was followed by a gradual increase towards prepartum luteolysis [102]. At the protein level, signals for Cx43 appeared diffusely in the placenta, and seemed to be more strongly targeted to maternal endothelium. At prepartum luteolysis, Cx43 appeared to be induced in the epithelial cells of superficial glands (i.e., glandular chambers). Interestingly, and in agreement with observations made in other species displaying invasive types of placentation, following implantation Cx26 appeared predominantly localized in the epithelial cells of superficial glands. Regarding possible

pregnancy-associated functions, Cx43 in humans appears to be essential for uterine angiogenesis and decidualization [109]. Additionally, as suggested by *in vitro* studies, Cx43 might regulate the differentiation of cytotrophoblast to syncytiotrophoblast and is involved in feto-maternal exchange [110]. Based on the observations presented herein on the spatio-temporal expression of Cx43 in canine uterus and placenta, we propose that this gap junction protein could be involved in uterine-placental angiogenesis and canine decidualization. The potential involvement of both connexins, Cx26 and Cx43, in placental feto-maternal communication at the time of prepartum luteolysis is also implied, but needs further verification.

Furthermore, the structural development of the canine endotheliochorial placenta has been, at least in part, investigated in this PhD work. In particular, possible mechanisms controlling the invasive behavior of the trophoblast were addressed. Accordingly, throughout all species invasive trophoblast behavior seems to be mediated by MMPs (reviewed in [111, 112]). With regard to the canine species, MMP2 and -9 have been detected in the trophoblast [89-91]. TIMPs are known to interact with MMPs, and as a consequence inhibit their biological functions [113]. Here, we investigated the spatio-temporal expression of TIMP2 and -4 in the dog. As mentioned elsewhere, we found increased mRNA levels of TIMP2 and -4 in response to the presence of free-floating embryos in the uterus [101, 102]. This increase might be correlated with increasing P4 levels, as well as with the decidualization process. This would resemble the situation in humans, where *in vitro* studies with endometrial cells revealed a positive association between expression of TIMPs and the decidualization process [114]. In addition, *in vitro* studies with a human trophoblastic cell line (i.e., BeWo cells) and rabbit uterine cervical fibroblasts indicated that TIMP2 expression is positively regulated by P4 [115, 116]. In the dog, as presented herein, a continuous gradual increase of both TIMPs was observed until pre-partum luteolysis [102]. Throughout gestation TIMP2 was detected in the myometrium and TIMP2 and -4 were detected around endometrial and placental blood vessels. Placental TIMP2 was distributed ubiquitously and was found in cytotrophoblast and syncytiotrophoblast, while TIMP4 was located only in cytotrophoblast. Interestingly, we found induced signals for TIMP2 and -4 in the endometrial connective tissue layer separating the superficial from deep glands, which attracted our attention [102]. The physiological function of this tissue layer as a possible barrier for the invading trophoblast has already been addressed elsewhere. However, when this barrier is breached, e.g., due to functional imbalances, excessive trophoblast invasion can proceed into the myometrium and result in SIPS [117]. As implied by their spatio-temporal expression, canine TIMP2 and -4 might be involved in protecting maternal uterine tissues from such excessive trophoblast invasion. It is not clear, however, if canine TIMP2 and TIMP4 interact with active canine MMP2 and MMP9, thus, this hypothesis needs further verification.

The second highest overrepresented functional term of the canine microarray analysis was associated with the immune system [101]. Indeed, immunomodulation, and thereby the

prevention of embryo rejection, is one of the first goals in embryo-maternal communication during early pregnancy in many species. Thus, e.g., in human pregnancy, the immune system is tightly regulated throughout gestation [118-120], with a specialized leukocyte fraction, so called uterine natural killer cells (uNK), being involved in vascular remodeling, angiogenesis and trophoblast invasion [121-123]. All these mechanisms are connected to decidualization.
The types and quantity of immune cells in the canine placenta still need to be elucidated. However, in the analyses we performed of the canine microarray, important immune system-related canonical pathways, such as complement system and pathways associated with adhesion and diapedesis of immune cells, or acute phase response signaling, were strongly represented [101]. Also, specific signaling pathways for interleukins (e.g., IL10, IL4 and IL6) appeared to be more strongly represented in the early pregnant pre-implantation uterus [101]. To verify the results from microarrays, highly conserved proteins with complex functions in the immune system, like AIF1 and IDO, were further investigated as possible candidate genes and were found to be differentially expressed in early pregnancy in response to the presence of embryos in the uterus [101]. With regard to genes with the highest individual fold-enrichment in response to the presence of free-floating embryos, it needs to be emphasized that, besides their involvement in ECM remodeling, ITIH4 and NOV are also linked to immune processes. Whereas NOV is involved in macrophage signaling [124, 125], ITIH4 as an acute phase protein is involved in systemic inflammation [126]. Interestingly, only ITIH4 was previously linked to reproductive functions in domestic animals, which certainly adds a novel aspect to comparative mammalian reproductive physiology. Furthermore, taking into account the immunomodulatory effects of free-floating embryos on the canine uterus, represented by the variety of induced genes, functional networks and pathways, it appears plausible that a functional intersection between these two systems (ECM and immune system) could be involved in the regulation of decidualization and placentation in the dog. In view of the limited invasiveness of canine trophoblast, this newly acquired knowledge could also lead to better understanding of different metabolic and immune system-controlled pathways involved in regulation of implantation in mammals. Together with some earlier studies, our microarray data convincingly confirmed involvement of the immune system during establishment of early pregnancy in the dog. More studies need, however, to be performed to understand the initiation of immunomodulation and its possible involvement in angiogenesis, trophoblast invasion, decidualization and tissue remodeling.

Next, the transcriptomic signatures of uterine gene expression characteristic of early, pre-implantation canine pregnancy were compared with genes expressed in uteri of other mammals during maternal recognition of pregnancy and exhibiting different types of placentation. Therefore, microarray data available from the literature for the early pregnant cow, sow, mare, and women during the window of implantation (WOI) were compared to the canine gene set [101]. The human WOI is a crucial time point, in which P4 and estrogen mediate morphological changes of the endometrium [127]. The outcome of this modification is the formation of maternal decidua and a receptive endometrium. An interesting finding

from this comparison was the fact that the canine gene set related to a higher degree with human genes than with those from other domestic animal species (cattle, horse or pig). Importantly, the high correlation observed between the canine and human gene set indicates that similar molecular systems with equal status of activation or deactivation (i.e., up- / downregulated) are utilized during early pregnancy. With this, indeed, the dog seems to be an interesting model for investigations on the early decidualization process in humans.

Another interesting observation derived from the inter-species comparisons of gene sets expressed in multiple species, including the dog, relates to the overall high number of genes that overlapped within all species when they were compared regardless of gene expression patterns (i.e., up- or down-regulated). Many of these gene sets showed opposite expression patterns, e.g., genes that were upregulated in the dog overlapped with down-regulated porcine genes and vice-versa [101]. This discovery indicates that implantation of conceptuses in different mammals relies on different strategies within similar regulatory pathways.
Interestingly, proenkephalin (PENK) and retinoic acid-metabolizing cytochrome (CYP26A1), were two genes represented in all compared species [101]. PENK, as part of the opioid system, seems to support embryonic and fetal growth by modulating analgesia and regulating uterine motility [128, 129], while CYP26A1 may be involved in regulating gene expression in embryonic and adult tissues [130, 131]. The functions of both genes in uterine preparation for implantation seem to be species-specific, and possibly also pregnancy stage-specific, as implied by their contrasting expression patterns among the different species [101].
Interestingly, the cumulative comparison of all genes not filtered for the direction of their expression (up- or down-regulated), revealed transcripts that did not overlap between dog and other species. Accordingly, 1926 genes were found exclusively expressed in the canine pre-implantation uterus. The functional annotation clustering revealed that these genes are associated with energy- and protein secretion-related functions [101]. It has been concluded that these exclusively regulated canine-specific genes should not be seen as an independent functional group, but rather as another subset of genes specifically altered during uterine remodeling and re-programming processes associated with species-specific early decidualization [101].

In summary:

(1) The canine embryo-maternal crosstalk in early (pre-attachment) pregnancy has been outlined utilizing a microarray approach. These data provided a unique basis for better understanding of the uterine milieu required for proper embryo implantation in dogs.

(2) Functional modification of ECM components apparently associated with early decidualization and preparation for implantation appears to be the main target of the embryo-derived signaling in the dog. Following the analysis of the microarray data, the uterine modulation of extracellular matrix has been investigated in more detail involving samples collected at selected stages of pregnancy, from early until late pregnancy. Starting with trophoblast attachment, morphological differentiation (decidualization) becomes apparent. At

later stages of pregnancy (i.e., following implantation and placentation) vigorous ECM remodeling occurs. This reflects dynamic feto-maternal interactions. Based on the expression of ECM components in the endometrial connective tissue layer the function of this layer as an active (and not only physical) barrier, protecting maternal tissues from (excessive) trophoblast invasion is suggested.

(3) The functional changes in the immune system commencing concomitantly with matrix remodeling are linked predominantly to: (i) the activation of phylogenetically conserved immunomodulatory proteins (e.g., AIF1 or IDO); (ii) the modulation of several chemokines (e.g., IPO, NOV and DIAPH); (iii) the involvement of multiple IL-signaling and activation of the complement system.

(4) Interspecies comparisons revealed that the dog could serve as an animal model in reproductive research and is worth attention; on the one hand it exhibits several reproductive peculiarities, on the other hand, however, it reveals some similarities, e.g., with regard to human uterine physiology during early pregnancy.

(5) With regard to PENK and CYP26A, our microarray analysis suggests their possible involvement in embryo-maternal communication across different species. Both genes have so far not been recognized as being important for reproductive events, but certainly deserve more attention in the future.

Decidualization in the dog

The unique process of canine uterine preparation for implantation has been, at least in part, previously investigated on the molecular level [29]. However, given that decidualization and embryo implantation demand uterine ECM modifications, a detailed description of the order of events (biochemical vs. morphological differentiation) involved in this species-specific tissue transformation process was missing so far for the dog.

In early pregnancy, free-floating embryos influence the biochemical behavior of the uterus in a paracrine manner [29, 101]. However, as clearly shown in the study presented herein involving samples from day 17 of pregnancy, which in the dog is the day of implantation [132, 133], the first morphological changes in the uterus can be observed only when the trophoblast germ layers attach to the uterine surface (*lamina epithelialis)* [103]. In this, canine decidualization resembles the embryo-induced process observed in rodents, rather than the hormonally-induced spontaneous decidualization known in humans [40, 41]. Following implantation, fully developed canine decidual cells are localized in close proximity to maternal vessels, indicating that, at least in part, their origin could be in the mesenchymal cells of *tunica media* layers of these vessels [103].

Due to the importance of decidualization for successful canine pregnancy, further investigations into the underlying regulatory mechanisms appear indispensable for more profound understanding of canine reproductive function. Thus, the additional goal of this PhD thesis was to improve our previously established *in vitro* decidualization protocol for canine uterine stromal cells [63]. That previous protocol utilized primary cell cultures that would

typically enter senescence after 2-3 passages (p). Consequently, due to restricted access to adequate tissue material, research possibilities were greatly limited. Therefore, since a more suitable cell culture model was absent, we decided to develop and characterize our own immortalized dog uterine stromal (DUS) cell line. A strategy involving transfection of cells with a plasmid vector carrying the large T-antigen (Tag) of simian vacuolating virus 40 (pSV40Tag) was successfully applied [103]. When the newly generated cell line was submitted to our standard decidualization protocol (0.5mM dibutyryl cyclic-AMP sodium salt (dbcAMP) over a time course of 72h), typical canine decidualization markers were up-regulated. The cells retained their biochemical and morphological characteristics over more than 30 passages. With this, the DUS cell line has been established as a unique and reliable canine *in vitro* decidualization model for further studies [103].

Using this model, we were able to address the important question of the capability of PGE2 to induce decidualization. Thus, we observed increased expression of PGR, PRLR, PTGES and PTGER4/EP4 in immortalized DUS cells undergoing PGE2-induced decidualization [103]. With this, in particular by showing the modulatory effects of PGE2 on PGR and PRLR expression, for the first time an indirect functional involvement of PGE2 in the P4-dependent establishment and maintenance of canine gestation, has been implicated [103]. Additionally, PGE2 seems to provide positive feedback on the expression of its own synthase (PTGES) and its receptor PTGER4/EP4.

In summary: these findings further strengthen the possible involvement of PGE2 in the canine decidualization process. The underlying molecular regulatory mechanisms of this event are currently under investigation; some preliminary results from this ongoing study are presented and discussed in the chapter *"Outlook: EP receptor blocking"*.

Further studies and outlook

Study 1: Blocking of EP receptors (EP2/4; PTGER2/4)

As mentioned elsewhere, the treatment of immortalized DUS cells with PGE2 affected the gene expression of decidualization markers, implying the involvement of PGE2 in canine decidualization. However, as yet the relative importance of PGE2 and its two cAMP-related receptors EP2/PTGER2 and EP4/PTGER4 during canine decidualization remains unexplored. Therefore, our ongoing experiments focus on EP2/PTGER2 receptor-mediated effects in PGE2-induced decidualization in canine immortalized DUS cells. For this, a specific blocker of EP2/PTGER2 (PF-04418948; Cayman Chemical Co., Ann Arbor, MI, USA) was used in dose-response experiments (0.1μM, 1.0μM and 10.0μM) in our 72h decidualization protocol. Prior to stimulation with PGE2, immortalized DUS cells were pre-incubated for 40 min with stimulation medium (as previously described in [103]: DMEM-High Glucose, pH 7.2-7.4, 0.1% bovine serum albumin (BSA), 100 U/ml penicillin, 100μg/ml streptomycin and 1% insulin-transferrin-selenium (ITS)) containing the EP2 blocker in various dosages. Following this, 10μM PGE2 was added to the culture medium for cells that had been pre-incubated with the blocker and then the cells were further incubated for 72h. This concentration of PGE2 was proved previously to efficiently induce decidualization in immortalized DUS cells [103]. For positive controls, cells were *in vitro* decidualized with PGE2 omitting the blocker. Untreated cells (i.e., cells incubated with stimulation medium without dbcAMP, PGE2 or EP2 blocker) served for negative controls. Morphological changes in cells were assessed using an inverted bright field microscope (Leica DM IL LED Fluo) and/or a live cell imaging microscope (LeicaDMI 6000B fluorescence microscope equipped with a Leica DFC360FX camera and automated stage). For live cell imaging, the automated stage system was used to record an image every 5 min from the same spot over 72h following our previously established protocol [63]. The viability and morphology of the immortalized DUS cells indicated no toxic effects of the EP2 blocker. Following the 72h treatment, cells were harvested and gene expression of selected decidualization markers was assessed. All experiments were repeated at least three times. GraphPad 3.06 software was used to test for statistical assumptions, such as normality and equality of variances. This was followed by a parametric one-way (ANOVA). In case of $P<0.05$, which was considered significant, the Tukey-Kramer multiple-comparisons test was applied. Numerical data are presented as geometric means (Xg) ± geometric standard deviation (S.D.). Whereas the expression of PGR and PRLR was significantly ($P<0.001$ and $P<0.05$, respectively; Fig. 1) increased following stimulation with PGE2, these effects were suppressed by applying the EP2 blocker. Thus, expression of PRLR was suppressed at $P<0.05$ when 1.0μM or 10.0μM EP2 blocker was applied; PGR expression decreased ($P<0.01$) following treatment with 1.0μM EP2 blocker, and at $P<0.001$ when 10μM EP2 blocker was applied (Fig. 1).

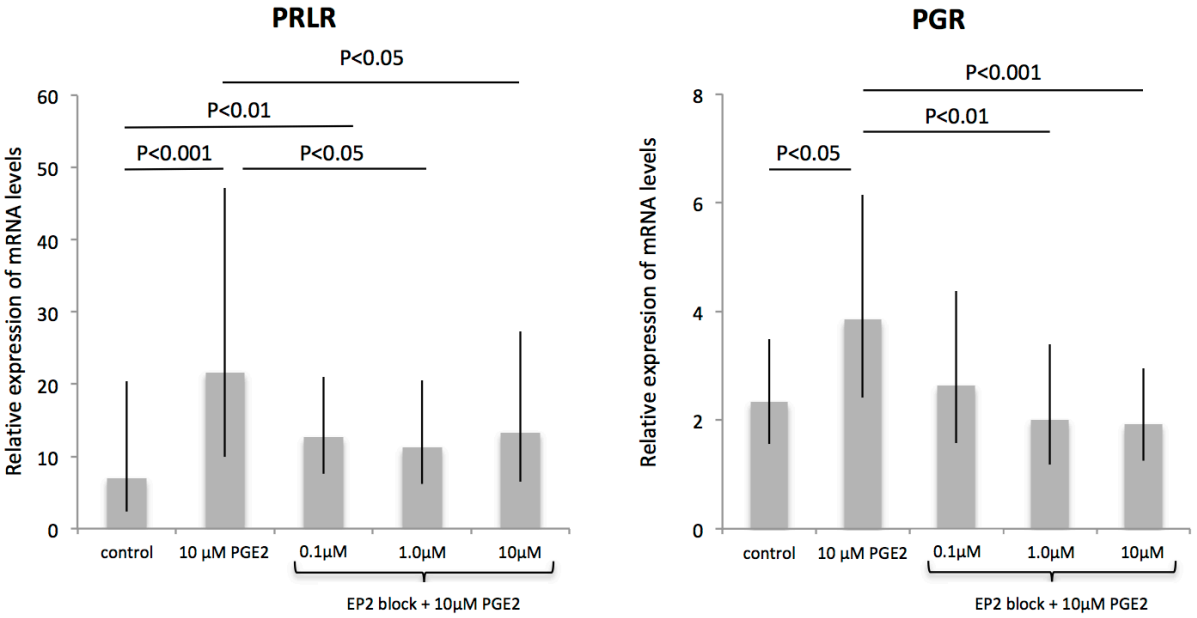


Figure 1: Expression of PRLR and PGR as determined by real time (TaqMan) PCR of immortalized DUS following treatment over 72h with 10μM PGE2 or 10μM PGE2 in the presence of the specific EP2 blocker (PF-04418948) (respective dosages are indicated). All experiments were repeated at least three times. Numerical data are presented as the geometric mean (Xg) ± geometric standard deviation (SD). A parametric one-way (ANOVA) was applied, followed by the Tukey-Kramer multiple-comparisons test. P<0.05 was considered significant. Detailed explanation is provided in the text

Cumulatively: Bearing in mind the fundamental role of decidual cells in the establishment and maintenance of canine pregnancy, these results suggest that the previously postulated [103] indirect functional involvement of embryonal and uterine PGE2 in the PGR- and PRLR-mediated effects in the canine uterus are EP2/PTGER2-dependent. Acting through this pathway, PGE2 appears to positively affect the local sensitivity of the canine uterus and placenta to P4 and PRL.

Further studies are planned investigating the potential involvement of EP4/PTGER4 in PGE2-mediated decidualization.

Study 2: Expression of genes encoding for selected ECM proteins during *in vitro* decidualization of canine uterine stroma cells

The microarray analysis of early pregnant uterine tissue indicated an alteration of the ECM prior to trophoblast adhesion and invasion [101]. Based on these results, further *in vivo* studies revealed that canine uterine ECM is moderately modified in early pregnancy, but undergoes vigorous reorganization processes within the uterus and placenta following implantation, which is associated with species-specific morphological decidualization [102]. Therefore, the presented ongoing study, utilizing both primary and immortalized DUS cells, assessed the expression of selected ECM proteins modulated during *in vivo* decidualization of the canine uterus. Cells (primary and immortalized DUS) were decidualized *in vitro* with our standardized protocol (0.5mM dbcAMP, 72h). The immortalized DUS cells were additionally stimulated with 10μM PGE2 to determine PGE2-mediated effects on ECM modulation. Experiments were performed on three consecutive passages (p) for immortalized DUS cells,

whereas p1 and p2 were used from four different primary DUS cell isolations (total n=8 experiments). For each cell culture (i.e., primary and immortalized DUS cells) negative controls consisted of omitting dbcAMP and/or PGE2 in the stimulation medium. Further controls consisted of immunofluorescence staining for VIMENTIN and pan-CYTOKERATIN. This was of particular importance for the primary cells, because VIMENTIN is an intermediate filament protein that can be used as a marker of mesenchymal type cells. Thereby, we were able to verify that the cell cultures consisted solely of fibroblast cells. Wide-spectrum CYTOKERATIN (Pan-CYTOKERATIN) staining was used for the detection of epithelial cells, which would indicate a spontaneous differentiation of the cell culture. The mesenchymal origin of primary and immortalized DUS cells was verified with nearly 100% cells staining positively for VIMENTIN in double-staining with wide-spectrum CYTOKERATIN.

Following cAMP/PGE2 decidualization of DUS cells, mRNA expression of the following ECM compounds was assessed by utilizing the semi-quantitative TaqMan PCR method: FN1, ECM1, LAMA2, Cx26, Cx43, TIMP2, TIMP4, COL1, COL3 and COL4. GraphPad 3.06 software was utilized for statistic evaluation. After testing for statistical assumptions, such as normality and equality of variances, a two-tailed Student´s t test was performed. $P<0.05$ was considered significant. Numerical data are presented as geometric means (Xg) ± geometric standard deviation (SD). The primary DUS cells responded to dbcAMP-induced decidualization with down-regulation of FN1 ($p=0.02$) and up-regulation of ECM1 and Cx43 ($P=0.01$ and $P=0.01$, respectively) (Fig. 2).

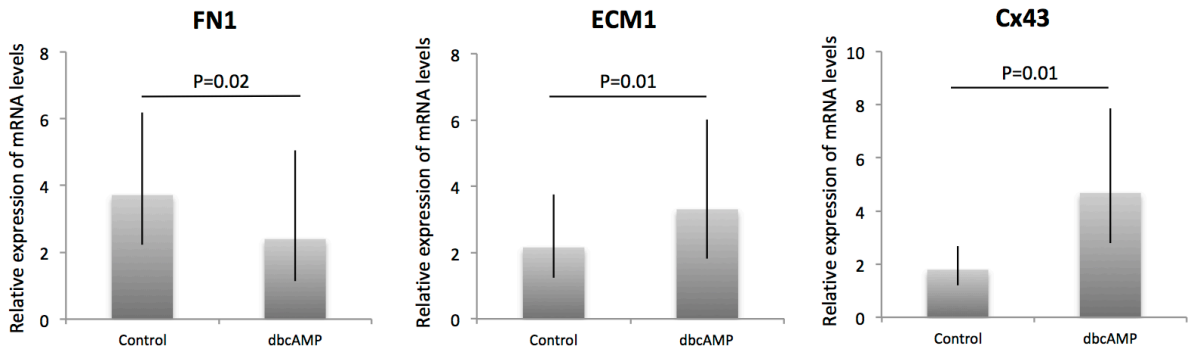


Figure 2: Expression of FN1, ECM1 and Cx43 in primary DUS cells following *in vitro* decidualization. Cells were treated with 0.5mM dbcAMP for 72h. Relative gene expression was determined by real time (TaqMan) PCR. All experiments were repeated at least three times. Numerical data are presented as geometric mean (Xg) ± geometric standard deviation (SD). An unpaired, two-tailed Student´s t-test was applied. Detailed explanation is provided in the text.

A broader spectrum of effects in response to dbcAMP was observed in the DUS cell line, which responded to treatment with dbcAMP by down-regulation of LAMA2 ($P<0.001$) and up-regulation of ECM1, Cx43 and COL4 ($P<0.001$, each) (Fig. 3). Expression of the respective proteins was visualized using immunofluorescence staining with the following antibodies: LAMA2 = Bioss Antibodies bs-8561R (dilution 1:200), ECM1 = Proteintech 11521-1-AP (dilution 1:100), Cx43 Abcam AB11370 (dilution 1:300), COL4 Abcam AB6586 (dilution 1:300). Detection of the respective ECM proteins was performed as described before

utilizing a LeicaDMI 6000B fluorescence microscope equipped with a Leica DFC360FX camera [63].

Stimulation with 10μM PGE2 had no effect on ECM protein mRNA levels of immortalized DUS cells (P>0.05, *not shown*).

In both cell culture systems (i.e., primary and immortalized DUS cells) 0.5mM dbcAMP did not affect gene expression of COL1, COL3, COL4, LAMA2, TIMP2, TIMP4 and Cx26 (P>0.5, *not shown*).

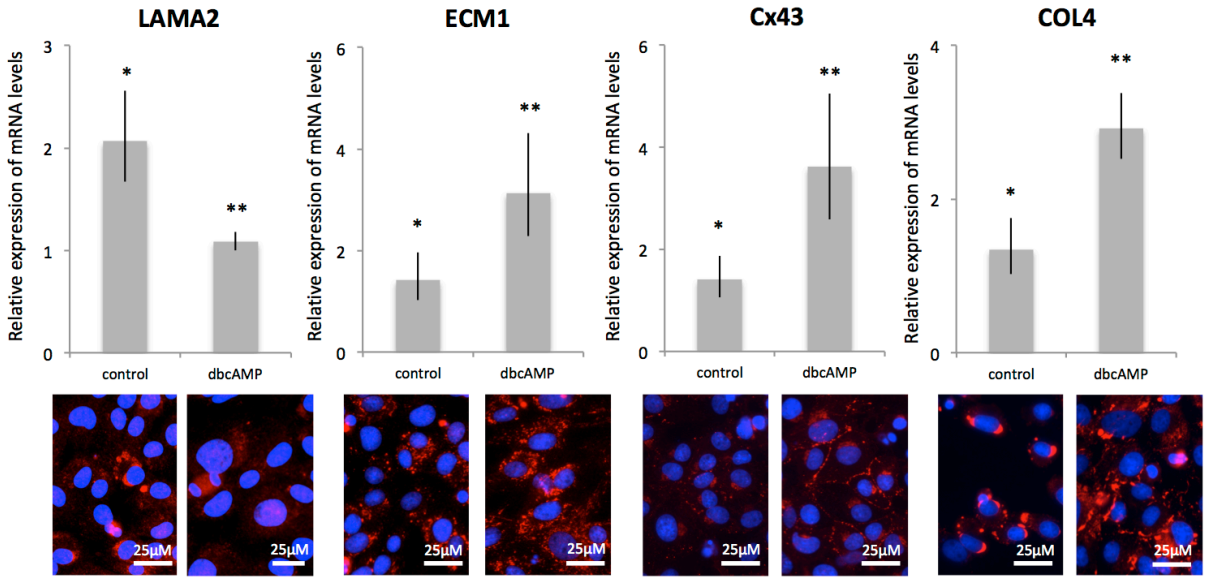


Figure 3: Expression of LAMA2, ECM1, Cx43 and COL4 in immortalized DUS cells during *in vitro* decidualization. Cells were treated with 0.5mM dbcAMP for 72h. Relative gene expression was determined by real time (TaqMan) PCR. Representative pictures of immunofluorescence staining show the localization and expression of ECM proteins (red staining). Nuclei were made visible with DAPI (blue staining). All experiments were repeated at least three times. Numerical data are presented as geometric means (Xg) ± geometric standard deviation (SD). An unpaired, two-tailed Student´s t-test was applied; asterisks (*) indicate P=0.001.

Cumulatively: These preliminary results indicate functional changes in the expression of specific ECM proteins during decidualization of canine uterine stromal cells. In particular, the molecular and biological functions of ECM1 during canine decidualization seem to be a worthwhile target for future studies as this gene was positively regulated in both cell culture systems (primary and immortalized DUS). Furthermore, the human ECM1 splice variant resulting in 1.8-kb transcript is predominantly found in placenta (and heart), indicating its involvement in reproductive functions [134].

As indicated elsewhere, this study is ongoing and further experiments are planned, in particular those aimed at assessing the expression levels of particular proteins.

Summary and outlook

Having identified the functional biological terms significantly affected by the presence of embryos in the canine early pregnant uterus, in this PhD work not only has an important knowledge gap been closed, but also a new research area has been launched regarding the establishment and maintenance of canine pregnancy. The information gained through our microarray approach can be used for future, more detailed studies. This certainly should

involve the role of the immune system in modulating canine uterine function throughout gestation. Indeed, in particular, there is scant information about leucocyte components in canine uterine tissue during pregnancy. In this regard, based on the IPA results presented herein (functional pathways analysis) some important pathways involving, e.g., IL-signaling or activation of the complement system, have been identified and certainly deserve more attention in future research. In mice and humans, the immune system is known to be involved in decidualization (reviewed in [40]), however, no such connection has yet been established for the dog. In contrast to mice, there are no available *in vivo* canine knockout models that would allow verification of these kinds of assumptions. However, the possible involvement of specific immune system-derived factors in the outcome of decidualization in the dog could be tested utilizing our newly established *in vitro* decidualization model with the immortalized DUS cell line generated in the course of the present PhD study.

These cells have been shown to constitute a unique model for *in vitro* investigations of canine decidualization. The capability of DUS cells to decidualize was clearly confirmed. Nevertheless, to obtain more detailed information about the species-specific decidualization process in the dog, a more comprehensive approach, involving for example, transcriptome analysis of immortalized DUS cells undergoing decidualization, should be considered. Such an approach would have the benefit of studying a single cell population, omitting the (background, noise) signals coming from other cell types.

Furthermore, considering the transcriptomic approach as a valuable research tool, future studies could be considered going beyond the time point of implantation and exploring further into advanced stages of pregnancy.

Presentation of data

The results described herein were presented at the following seminars and conferences:

June 19th 2015: Global transcriptomic analysis and profiling of the canine early pregnant, pre-implantation uterus, and expression and distribution patterns of selected extracellular matrix proteins (oral presentation); Annual Meeting of the Society for the Study of Reproduction (SSR); San Juan, PR, USA

September 3rd 2015: PhD-Introduction and pilot results (oral presentation): Institute of Veterinary Anatomy, University of Zurich, Switzerland

October 2nd 2015: Global transcriptomic analysis and profiling of the canine early pregnant, pre-implantation uterus, and expression and distribution patterns of selected extracellular matrix proteins (oral presentation); (Annual Meeting) Pregnancy, Parturition, Post-partum (PPP)-meeting, Veterinary University Vienna, Vienna, Austria

August 20th 2015: Global transcriptomic analysis and profiling of the canine early pregnant, pre-implantation uterus, and expression and distribution patterns of selected extracellular matrix proteins (oral presentation); Science and BBQ, Vetsuisse Faculty Bern, Switzerland

December 7th 2015: Global transcriptomic analysis, expression and distribution patterns of selected uterine extracellular matrix proteins during the onset and maintenance of canine pregnancy (oral presentation); Monday Seminar at the ETH Zurich, Institute of Agricultural Sciences, Zurich, Switzerland

February 4th 2016: Spatio-temporal uterine expression of extracellular matrix proteins during the embryonal pre-attachment phase and selected stages of canine pregnancy following implantation (poster presentation); GCB students symposium Bern, Switzerland

February 11th 2016: Expression and distribution patterns of selected extracellular matrix proteins during the onset and maintenance of pregnancy in the canine uterus (poster presentation) no. 49; Jahrestagung der Physiologie und Pathologie der Fortpflanzung (Februartagung), Veterinärmedizinische Fakultät Leipzig, Germany

June 25th 2016: In vivo and in vitro decidualization of the canine uterus (invited talk); The 8th quadrennial international symposium on canine and feline reproduction (ISCFR) in a joint meeting with the 19th European Veterinary Society for Small Animal Reproduction Congress (EVSSAR), Paris, France

October 14th 2016: Morpho-functional aspects of canine uterine decidualization: in vivo observations and in-vitro model (oral presentation); Center for Applied Biotechnology and Molecular Medicine (CABMM)-Lecture-Series Vetsuisse Zurich, University of Zurich, Zurich, Switzerland

December 5th 2016: Decidualization in mammals: comparative aspects and canine specific regulatory mechanisms (oral presentation); Monday Seminar at the ETH Zurich, Institute of Agricultural Sciences, Zurich, Switzerland

February 2nd 2017: In vitro study on canine decidualization: role and functional characterization of decidualization using immortalized canine uterine stromal cell lines (poster presentation); GCB students symposium, Bern, Switzerland

July 10th 2017: Signaling pathways and immune system during decidualization (oral presentation); Institute of Veterinary-Anatomy Zurich, Zurich, Switzerland

July 13-16th 2017: PGE2-dependent, PTGER2/EP2 receptor-mediated effects on canine decidualization using an in vitro model of immortalized canine uterine stromal cells (poster presentation); Annual meeting of the Society for the Study of Reproduction (SSR) Washington DC, USA

September 30th 2017: Embryo-maternal crosstalk (oral presentation); Annual Meeting of Gonadal Function, Gamete Interaction and Pregnancy (GGP) (former name: pregnancy, parturition, post partum (PPP)-meeting), Justus-Liebig-University Gießen, Germany

Reference List

1. Bazer, F.W., *History of Maternal Recognition of Pregnancy*. Adv Anat Embryol Cell Biol, 2015. **216**: p. 5-25.
2. Short, R.V., *Implantation and the Maternal Recognition of Pregnancy*, in *Ciba Foundation Symposium - Foetal Autonomy*, O.C. M, Editor. 1969, Wolstenholme GEW: Churchill, London. p. 2-31.
3. Bazer, F.W., et al., *Physiological mechanisms of pregnancy recognition in ruminants*. J Reprod Fertil Suppl, 1991. **43**: p. 39-47.
4. Spencer, T.E. and F.W. Bazer, *Conceptus signals for establishment and maintenance of pregnancy*. Reprod Biol Endocrinol, 2004. **2**: p. 49.
5. Roberts, R.M., et al., *Evolution of the interferon tau genes and their promoters, and maternal-trophoblast interactions in control of their expression*. Reprod Suppl, 2003. **61**: p. 239-51.
6. Perry, J.S., R.B. Heap, and E.C. Amoroso, *Steroid hormone production by pig blastocysts*. Nature, 1973. **245**(5419): p. 45-7.
7. Bazer, F.W. and W.W. Thatcher, *Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin F2alpha by the uterine endometrium*. Prostaglandins, 1977. **14**(2): p. 397-400.
8. Sharp, D.C., et al., *The continuum of events leading to maternal recognition of pregnancy in mares*. J Reprod Fertil Suppl, 1989. **37**: p. 101-7.
9. Sharp, D.C., *The early fetal life of the equine conceptus*. Anim Reprod Sci, 2000. **60-61**: p. 679-89.
10. Boerboom, D., et al., *Expression of key prostaglandin synthases in equine endometrium during late diestrus and early pregnancy*. Biol Reprod, 2004. **70**(2): p. 391-9.
11. Concannon, P.W., J.P. McCann, and M. Temple, *Biology and endocrinology of ovulation, pregnancy and parturition in the dog*. J Reprod Fertil Suppl, 1989. **39**: p. 3-25.
12. Kowalewski, M.P., et al., *The Dog: Nonconformist, Not Only in Maternal Recognition Signaling*. Adv Anat Embryol Cell Biol, 2015. **216**: p. 215-37.
13. Steinetz, B.G., et al., *Serum relaxin and progesterone concentrations in pregnant, pseudopregnant, and ovariectomized, progestin-treated pregnant bitches: detection of relaxin as a marker of pregnancy*. Am J Vet Res, 1989. **50**(1): p. 68-71.
14. Kowalewski, M.P., *Luteal regression vs. prepartum luteolysis: regulatory mechanisms governing canine corpus luteum function*. Reprod Biol, 2014. **14**(2): p. 89-102.
15. Kowalewski, M.P., *Endocrine and molecular control of luteal and placental function in dogs: a review*. Reprod Domest Anim, 2012. **47 Suppl 6**: p. 19-24.
16. Onclin, K., B. Murphy, and J.P. Verstegen, *Comparisons of estradiol, LH and FSH patterns in pregnant and nonpregnant beagle bitches*. Theriogenology, 2002. **57**(8): p. 1957-72.
17. Hoffmann, B., et al., *Investigations on hormonal changes around parturition in the dog and the occurrence of pregnancy-specific non conjugated oestrogens*. Exp Clin Endocrinol, 1994. **102**(3): p. 185-9.
18. Nowak, M., et al., *Functional implications of the utero-placental relaxin (RLN) system in the dog throughout pregnancy and at term*. Reproduction, 2017. **154**(4): p. 415-431.
19. Bukowska, D., et al., *Analysis of integrins and vascular endothelial growth factor isoforms mRNA expression in the canine uterus during perimplantation period*. Pol J Vet Sci, 2011. **14**(2): p. 253-8.
20. Schafer-Somi, S., et al., *Vascular endothelial (VEGF) and epithelial growth factor (EGF) as well as platelet-activating factor (PAF) and receptors are expressed in the early pregnant canine uterus*. Reprod Domest Anim, 2013. **48**(1): p. 20-6.
21. Plaisier, M., *Decidualisation and angiogenesis*. Best Pract Res Clin Obstet Gynaecol, 2011. **25**(3): p. 259-71.
22. Demir, R., A. Yaba, and B. Huppertz, *Vasculogenesis and angiogenesis in the endometrium during menstrual cycle and implantation*. Acta Histochem, 2010. **112**(3): p. 203-14.
23. Lima, P.D., et al., *Leukocyte driven-decidual angiogenesis in early pregnancy*. Cell Mol Immunol, 2014. **11**(6): p. 522-37.
24. Eckersall, P.D., et al., *Acute phase proteins in canine pregnancy (Canis familiaris)*. J Reprod Fertil Suppl, 1993. **47**: p. 159-64.
25. Concannon, P.W., et al., *Postimplantation increase in plasma fibrinogen concentration with increase in relaxin concentration in pregnant dogs*. Am J Vet Res, 1996. **57**(9): p. 1382-5.
26. Schafer-Somi, S., et al., *Expression of genes in the canine pre-implantation uterus and embryo: implications for an active role of the embryo before and during invasion*. Reprod Domest Anim, 2008. **43**(6): p. 656-63.
27. Beceriklisoy, H.B., et al., *Cytokines, growth factors and prostaglandin synthesis in the uterus of pregnant and non-pregnant bitches: the features of placental sites*. Reprod Domest Anim, 2009. **44 Suppl 2**: p. 115-9.
28. Pennington, K.A., et al., *Preeclampsia: multiple approaches for a multifactorial disease*. Dis Model Mech, 2012. **5**(1): p. 9-18.
29. Kautz, E., et al., *Expression of genes involved in the embryo-maternal interaction in the early-pregnant canine uterus*. Reproduction, 2014. **147**(5): p. 703-17.
30. Schafer-Somi, S., *The pregnant canine uterus: molecular insights until placentation*. Reproduction in domestic animals, 2011. **46**(Suppl 3): p. 62.

31. Emera, D., R. Romero, and G. Wagner, *The evolution of menstruation: a new model for genetic assimilation: explaining molecular origins of maternal responses to fetal invasiveness*. Bioessays, 2012. **34**(1): p. 26-35.
32. Brosens, J.J., et al., *A role for menstruation in preconditioning the uterus for successful pregnancy*. Am J Obstet Gynecol, 2009. **200**(6): p. 615.e1-6.
33. Wislocki, G.B. and E.W. Dempsey, *Histochemical reactions in the placenta of the cat*. Am J Anat, 1946. **78**: p. 1-45.
34. Dempsey, E.W. and G.B. Wislocki, *Electron microscopic observations on the placenta of the cat*. J Biophys Biochem Cytol, 1956. **2**(6): p. 743-54.
35. Cha, J., X. Sun, and S.K. Dey, *Mechanisms of implantation: strategies for successful pregnancy*. Nat Med, 2012. **18**(12): p. 1754-67.
36. Teles, A. and A.C. Zenclussen, *How cells of the immune system prepare the endometrium for implantation*. Semin Reprod Med, 2014. **32**(5): p. 358-64.
37. Damsky, C., A. Sutherland, and S. Fisher, *Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation*. Faseb j, 1993. **7**(14): p. 1320-9.
38. Smith, S.D., et al., *Changes in vascular extracellular matrix composition during decidual spiral arteriole remodeling in early human pregnancy*. Histol Histopathol, 2016. **31**(5): p. 557-71.
39. Lala, P.K. and P. Nandi, *Mechanisms of trophoblast migration, endometrial angiogenesis in preeclampsia: The role of decorin*. Cell Adh Migr, 2016. **10**(1-2): p. 111-25.
40. Gellersen, B. and J.J. Brosens, *Cyclic decidualization of the human endometrium in reproductive health and failure*. Endocr Rev, 2014. **35**(6): p. 851-905.
41. Gellersen, B., I.A. Brosens, and J.J. Brosens, *Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives*. Semin Reprod Med, 2007. **25**(6): p. 445-53.
42. Telgmann, R. and B. Gellersen, *Marker genes of decidualization: activation of the decidual prolactin gene*. Hum Reprod Update, 1998. **4**(5): p. 472-9.
43. Hunter, W., *The anatomy of the human gravid uterus*. London, 1774.
44. Thornton, J.L. and P.C. Want, *William Hunter's "The anatomy of the human gravid uterus" 1774-1974*. J Obstet Gynaecol Br Commonw, 1974. **81**(1): p. 1-10.
45. Abrahamsohn, P.A. and T.M. Zorn, *Implantation and decidualization in rodents*. J Exp Zool, 1993. **266**(6): p. 603-28.
46. Arnold, G. and T.H. Schiebler, *Lehrbuch der gesamten Anatomie des Menschen: Cytologie, Histologie, Entwicklungsgeschichte, makroskopische und mikroskopische Anatomie*. 2013: Springer Berlin Heidelberg.
47. Schnorr, B. and M. Kressin, *Embryologie der Haustiere: ein Kurzlehrbuch*. 2006: Enke.
48. Telgmann, R., et al., *Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells*. Endocrinology, 1997. **138**(3): p. 929-37.
49. Gellersen, B. and J. Brosens, *Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair*. J Endocrinol, 2003. **178**(3): p. 357-72.
50. de Ziegler, D., et al., *The hormonal control of endometrial receptivity: estrogen (E2) and progesterone*. J Reprod Immunol, 1998. **39**(1-2): p. 149-66.
51. van den Heuvel, M.J., et al., *Trafficking of circulating pro-NK cells to the decidualizing uterus: regulatory mechanisms in the mouse and human*. Immunol Invest, 2005. **34**(3): p. 273-93.
52. Renthal, N.E., et al., *Molecular Regulation of Parturition: A Myometrial Perspective*. Cold Spring Harb Perspect Med, 2015. **5**(11).
53. Pineda-Torres, M., et al., *Evidence of an immunosuppressive effect of progesterone upon in vitro secretion of proinflammatory and prodegradative factors in a model of choriodecidual infection*. Bjog, 2015. **122**(13): p. 1798-807.
54. Binart, N., et al., *Rescue of preimplantatory egg development and embryo implantation in prolactin receptor-deficient mice after progesterone administration*. Endocrinology, 2000. **141**(7): p. 2691-7.
55. Jabbour, H.N., H.O. Critchley, and S.C. Boddy, *Expression of functional prolactin receptors in nonpregnant human endometrium: janus kinase-2, signal transducer and activator of transcription-1 (STAT1), and STAT5 proteins are phosphorylated after stimulation with prolactin*. J Clin Endocrinol Metab, 1998. **83**(7): p. 2545-53.
56. Kowalewski, M.P., et al., *Luteal and placental function in the bitch: spatio-temporal changes in prolactin receptor (PRLr) expression at dioestrus, pregnancy and normal and induced parturition*. Reprod Biol Endocrinol, 2011. **9**: p. 109.
57. Concannon, P.W., *Endocrinologic control of normal canine ovarian function*. Reprod Domest Anim, 2009. **44 Suppl 2**: p. 3-15.
58. Kowalewski, M.P., et al., *Canine placenta: a source of prepartal prostaglandins during normal and antiprogestin-induced parturition*. Reproduction, 2010. **139**(3): p. 655-64.
59. Vermeirsch, H., P. Simoens, and H. Lauwers, *Immunohistochemical detection of the estrogen receptor-alpha and progesterone receptor in the canine pregnant uterus and placental labyrinth*. Anat Rec, 2000. **260**(1): p. 42-50.
60. Gram, A., et al., *Biosynthesis and degradation of canine placental prostaglandins: prepartum changes in expression and function of prostaglandin F2alpha-synthase (PGFS, AKR1C3) and 15-hydroxyprostaglandin dehydrogenase (HPGD)*. Biol Reprod, 2013. **89**(1): p. 2.
61. Gram, A., et al., *Canine placental prostaglandin E2 synthase: expression, localization, and biological functions in providing substrates for prepartum PGF2alpha synthesis*. Biol Reprod, 2014. **91**(6): p. 154.
62. Gram, A., A. Boos, and M.P. Kowalewski, *Uterine and placental expression of canine oxytocin receptor during pregnancy and normal and induced parturition*. Reprod Domest Anim, 2014. **49 Suppl 2**: p. 41-9.

63. Kautz, E., et al., *In vitro decidualisation of canine uterine stromal cells*. Reprod Biol Endocrinol, 2015. **13**: p. 85.
64. Ricciotti, E. and G.A. FitzGerald, *Prostaglandins and Inflammation*. Arterioscler Thromb Vasc Biol, 2011. **31**(5): p. 986-1000.
65. Simmons, D.L., R.M. Botting, and T. Hla, *Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition*. Pharmacol Rev, 2004. **56**(3): p. 387-437.
66. Kennedy, T.G., C. Gillio-Meina, and S.H. Phang, *Prostaglandins and the initiation of blastocyst implantation and decidualization*. Reproduction, 2007. **134**(5): p. 635-43.
67. Lim, H., et al., *Multiple female reproductive failures in cyclooxygenase 2-deficient mice*. Cell, 1997. **91**(2): p. 197-208.
68. Miller, M.M. and C.C. O'Morchoe, *Decidual cell reaction induced by prostaglandin F2 alpha in the mature oophorectomized rat*. Cell Tissue Res, 1982. **225**(1): p. 189-99.
69. Asselin, E. and M.A. Fortier, *Detection and regulation of the messenger for a putative bovine endometrial 9-keto-prostaglandin E(2) reductase: effect of oxytocin and interferon-tau*. Biol Reprod, 2000. **62**(1): p. 125-31.
70. Woodward, D.F., R.L. Jones, and S. Narumiya, *International Union of Basic and Clinical Pharmacology. LXXXIII: Classification of Prostanoid Receptors, Updating 15 Years of Progress*. Pharmacological Reviews, 2011. **63**(3): p. 471.
71. Brosens, J.J., N. Hayashi, and J.O. White, *Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells*. Endocrinology, 1999. **140**(10): p. 4809-20.
72. Frank, G.R., et al., *Prostaglandin E2 enhances human endometrial stromal cell differentiation*. Endocrinology, 1994. **134**(1): p. 258-63.
73. Kennedy, T.G. and P.E. Doktorcik, *Effects of analogues of prostaglandin E2 and F2 alpha on the decidual cell reaction in the rat*. Prostaglandins, 1988. **35**(2): p. 207-19.
74. Yee, G.M. and T.G. Kennedy, *Prostaglandin E2, cAMP and cAMP-dependent protein kinase isozymes during decidualization of rat endometrial stromal cells in vitro*. Prostaglandins, 1993. **46**(2): p. 117-38.
75. Stys, S.J., et al., *Effect of prostaglandin E2 on cervical compliance in pregnant ewes*. Am J Obstet Gynecol, 1981. **140**(4): p. 415-9.
76. Fuchs, A.R., et al., *Cervical ripening and plasma prostaglandin levels. Comparison of endocervical and extra-amniotic PGE2*. Prostaglandins, 1984. **28**(2): p. 217-27.
77. Kowalewski, M.P., et al., *Prostaglandin E2 functions as a luteotrophic factor in the dog*. Reproduction, 2013. **145**(3): p. 213-26.
78. Brar, A.K., et al., *Progesterone-dependent decidualization of the human endometrium is mediated by cAMP*. Endocrine, 1997. **6**(3): p. 301-7.
79. Parhar, R.S., S. Yagel, and P.K. Lala, *PGE2-mediated immunosuppression by first trimester human decidual cells blocks activation of maternal leukocytes in the decidua with potential anti-trophoblast activity*. Cell Immunol, 1989. **120**(1): p. 61-74.
80. Hubmacher, D. and S.S. Apte, *The biology of the extracellular matrix: novel insights*. Curr Opin Rheumatol, 2013. **25**(1): p. 65-70.
81. da Anunciacao, A., et al., *Extracellular matrix in epitheliochorial, endotheliochorial and haemochorial placentation and its potential application for regenerative medicine*. Reprod Domest Anim, 2017. **52**(1): p. 3-15.
82. Daley, W.P. and K.M. Yamada, *ECM-modulated cellular dynamics as a driving force for tissue morphogenesis*. Curr Opin Genet Dev, 2013. **23**(4): p. 408-14.
83. Hjelm, A.M., et al., *Changes of the uterine proteoglycan distribution at term pregnancy and during labour*. Eur J Obstet Gynecol Reprod Biol, 2002. **100**(2): p. 146-51.
84. Leoni, P., F. Carli, and D. Halliday, *Collagen in the Pregnant and Non Pregnant Uterus*. Clinical Science, 1990. **79**(s23): p. 10P-11P.
85. Cabrol, D., et al., *Pregnancy-related changes in the distribution of glycosaminoglycans in the cervix and corpus of the human uterus*. Eur J Obstet Gynecol Reprod Biol, 1985. **20**(5): p. 289-95.
86. Boos, A., A. Stelljes, and J. Kohtes, *Collagen types I, III and IV in the placentome and interplacentomal maternal and fetal tissues in normal cows and in cattle with retention of fetal membranes*. Cells Tissues Organs, 2003. **174**(4): p. 170-83.
87. Bischof, P., et al., *Expression of extracellular matrix-degrading metalloproteinases by cultured human cytotrophoblast cells: effects of cell adhesion and immunopurification*. Am J Obstet Gynecol, 1991. **165**(6 Pt 1): p. 1791-801.
88. Bischof, P., et al., *Importance of matrix metalloproteinases in human trophoblast invasion*. Early Pregnancy, 1995. **1**(4): p. 263-9.
89. Diessler, M., et al., *Differential expression and activity of matrix metalloproteinases 2 and 9 in canine early placenta*. Reprod Domest Anim, 2017. **52**(1): p. 35-43.
90. Beceriklisoy, H.B., et al., *Matrix metalloproteinase (MMP)-2 and MMP-9 activity in the canine uterus before and during placentation*. Reprod Domest Anim, 2007. **42**(6): p. 654-9.
91. Fellows, E.J., T.M. Hazzard, and M.A. Kutzler, *Gene expression in pre-term, pre-labour and parturient canine placenta*. Reprod Domest Anim, 2012. **47 Suppl 6**: p. 182-5.
92. Wewer, U.M., et al., *Mouse endometrial stromal cells produce basement-membrane components*. Differentiation, 1986. **32**(1): p. 49-58.
93. Wewer, U.M., et al., *Immunochemical and ultrastructural assessment of the nature of the pericellular basement membrane of human decidual cells*. Lab Invest, 1985. **53**(6): p. 624-33.
94. Sharkey, A.M. and N.S. Macklon, *The science of implantation emerges blinking into the light*. Reprod Biomed Online, 2013. **27**(5): p. 453-60.
95. Johnson, G.A., et al., *Osteopontin: roles in implantation and placentation*. Biol Reprod, 2003. **69**(5): p. 1458-71.

96. Brown, J.J. and V.E. Papaioannou, *Distribution of hyaluronan in the mouse endometrium during the periimplantation period of pregnancy*. Differentiation, 1992. **52**(1): p. 61-8.
97. Clark, D.E., et al., *Immunolocalization of collagen type I and laminin in the uterus on days 5 to 8 of embryo implantation in the rat*. Anat Rec, 1993. **237**(1): p. 8-20.
98. Morrione, T.G. and S. Seifter, *Alteration in the collagen content of the human uterus during pregnancy and post partum involution*. J Exp Med, 1962. **115**: p. 357-65.
99. Schwalm, H. and K. Cretius, *[Content of contractile proteins, water-soluble proteins and stroma proteins in the uterine musculature]*. Arch Gynakol, 1958. **191**(3): p. 271-82.
100. Cohen, M. and P. Bischof, *Factors regulating trophoblast invasion*. Gynecol Obstet Invest, 2007. **64**(3): p. 126-30.
101. Graubner, F.R., et al., *Uterine responses to early pre-attachment embryos in the domestic dog and comparisons with other domestic animal species*. Biol Reprod, 2017. **97**(2): p. 197-216.
102. Graubner, F.R., et al., *Uterine and placental distribution of selected extracellular matrix (ECM) components in the dog*. Reproduction, 2018. **155**: p. 403-421.
103. Graubner, F.R., et al., *Decidualization of the canine uterus: From early until late gestational in vivo morphological observations, and functional characterization of immortalized canine uterine stromal cell lines*. Reprod Domest Anim, 2017. **52 Suppl 2**: p. 137-147.
104. Winterhager, E., et al., *Spatial and temporal expression of connexin26 and connexin43 in rat endometrium during trophoblast invasion*. Dev Biol, 1993. **157**(2): p. 399-409.
105. Grummer, R., et al., *Regulation of connexin26 and connexin43 expression in rat endometrium by ovarian steroid hormones*. Biol Reprod, 1994. **51**(6): p. 1109-16.
106. Johnson, M.L., et al., *Gap junctional connexin messenger RNA expression in the ovine uterus and placenta: effects of estradiol-17beta-treatment, early pregnancy stages, and embryo origin*. Domest Anim Endocrinol, 2017. **58**: p. 104-112.
107. Grummer, R., et al., *Different regulatory pathways of endometrial connexin expression: preimplantation hormonal-mediated pathway versus embryo implantation-initiated pathway*. Biol Reprod, 2004. **71**(1): p. 273-81.
108. Grummer, R., B. Reuss, and E. Winterhager, *Expression pattern of different gap junction connexins is related to embryo implantation*. Int J Dev Biol, 1996. **40**(1): p. 361-7.
109. Laws, M.J., et al., *Gap junction communication between uterine stromal cells plays a critical role in pregnancy-associated neovascularization and embryo survival*. Development, 2008. **135**(15): p. 2659-68.
110. Cronier, L., et al., *Connexin expression and gap junctional intercellular communication in human first trimester trophoblast*. Mol Hum Reprod, 2002. **8**(11): p. 1005-13.
111. Goldman-Wohl, D. and S. Yagel, *Regulation of trophoblast invasion: from normal implantation to pre-eclampsia*. Mol Cell Endocrinol, 2002. **187**(1-2): p. 233-8.
112. Cohen, M., A. Meisser, and P. Bischof, *Metalloproteinases and human placental invasiveness*. Placenta, 2006. **27**(8): p. 783-93.
113. Brew, K. and H. Nagase, *The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity*. Biochim Biophys Acta, 2010. **1803**(1): p. 55-71.
114. Graham, A., J. Holbert, and W.B. Nothnick, *miR-181b-5p Modulates Cell Migratory Proteins, Tissue Inhibitor of Metalloproteinase 3, and Annexin A2 During In Vitro Decidualization in a Human Endometrial Stromal Cell Line*. Reprod Sci, 2017. **24**(9): p. 1264-1274.
115. Imada, K., et al., *Progesterone increases the production of tissue inhibitor of metalloproteinases-2 in rabbit uterine cervical fibroblasts*. FEBS Lett, 1994. **341**(1): p. 109-12.
116. Jo, Y.S., et al., *Progesterone Inhibits Leptin-Induced Invasiveness of BeWo Cells*. Int J Med Sci, 2015. **12**(10): p. 773-9.
117. Al-Bassam, M.A., R.G. Thomson, and L. O'Donnell, *Involution abnormalities in the postpartum uterus of the bitch*. Vet Pathol, 1981. **18**(2): p. 208-18.
118. Taylor, E.B. and J.M. Sasser, *Natural killer cells and T lymphocytes in pregnancy and pre-eclampsia*. Clin Sci (Lond), 2017. **131**(24): p. 2911-2917.
119. Liu, S., et al., *The role of decidual immune cells on human pregnancy*. J Reprod Immunol, 2017. **124**: p. 44-53.
120. Le Bouteiller, P. and A. Bensussan, *Up-and-down immunity of pregnancy in humans*. F1000Res, 2017. **6**: p. 1216.
121. Ashkar, A.A., J.P. Di Santo, and B.A. Croy, *Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy*. J Exp Med, 2000. **192**(2): p. 259-70.
122. Monk, J.M., et al., *Induction of murine spiral artery modification by recombinant human interferon-gamma*. Placenta, 2005. **26**(10): p. 835-8.
123. Hanna, J., et al., *Decidual NK cells regulate key developmental processes at the human fetal-maternal interface*. Nat Med, 2006. **12**(9): p. 1065-74.
124. Chen, P.C., et al., *Prostate cancer-derived CCN3 induces M2 macrophage infiltration and contributes to angiogenesis in prostate cancer microenvironment*. Oncotarget, 2014. **5**(6): p. 1595-608.
125. Shi, H., et al., *CCN3 Regulates Macrophage Foam Cell Formation and Atherosclerosis*. Am J Pathol, 2017. **187**(6): p. 1230-1237.
126. Gonzalez-Ramon, N., et al., *The major acute phase serum protein in pigs is homologous to human plasma kallikrein sensitive PK-120*. FEBS Lett, 1995. **371**(3): p. 227-30.
127. Altmae, S., et al., *Research resource: interactome of human embryo implantation: identification of gene expression pathways, regulation, and integrated regulatory networks*. Mol Endocrinol, 2012. **26**(1): p. 203-17.
128. Jin, D.F., et al., *Estrous cycle- and pregnancy-related differences in expression of the proenkephalin and proopiomelanocortin genes in the ovary and uterus*. Endocrinology, 1988. **122**(4): p. 1466-71.

129. Zhu, Y. and J.E. Pintar, *Expression of opioid receptors and ligands in pregnant mouse uterus and placenta*. Biol Reprod, 1998. **59**(4): p. 925-32.
130. Han, B.C., et al., *Retinoic acid-metabolizing enzyme cytochrome P450 26a1 (cyp26a1) is essential for implantation: functional study of its role in early pregnancy*. J Cell Physiol, 2010. **223**(2): p. 471-9.
131. Xia, H.F., et al., *Retinoic acid metabolizing enzyme CYP26A1 is implicated in rat embryo implantation*. Hum Reprod, 2010. **25**(12): p. 2985-98.
132. Amoroso, E.C., *Placentation*. 1952, Marshall's physiology of reproduction.: Longmans Green, London.
133. Kehrer, A., *[Chorionic development and structure of the placenta zonaria in the cat, dog and fox (author's transl)]*. Z Anat Entwicklungsgesch, 1973. **143**(1): p. 25-42.
134. Smits, P., et al., *The human extracellular matrix gene 1 (ECM1): genomic structure, cDNA cloning, expression pattern, and chromosomal localization*. Genomics, 1997. **45**(3): p. 487-95.

Curriculum vitae and list of publications

Curriculum Vitae

NAME: Felix Reinhold (Manfred) Graubner
ADDRESS 1: Silberstr. 46, 30655, Hannover, Germany
ADDRESS 2: Frohburgstr. 283, 8057, Zürich, Switzerland
DATE OF BIRTH: August 12th, 1983
NATIONALITY: German
EMAIL: Felix.graubner@vetanat.uzh.ch, felixgraubner@gmx.de

Education

April 2014 to present: University of Zurich, Vetsuisse Faculty, Institute of Veterinary Anatomy, Switzerland
PhD student in the research group of Prof. Dr. MP Kowalewski, PhD

2008-2014: University of Veterinary Medicine Hannover, Germany
Diploma in Veterinary Medicine

2006-2008: University of Veterinary Medicine Hannover, Germany
Education as Veterinary Medical Technician
(not completed in favor of study)

2005: BSA Academy, School for Health Management
Correspondence course as fitness instructor, B-License, basic qualification DIN-Norm 33961 (Stage 2)

2001-2003: Leibnizgymnasium (grammar school) Hannover, Germany
A-Levels in Chemistry and English

2000-2001: High School Vassar Michigan,
USA High School diploma

1996-2000: Leibnizgymnasium (secondary education) Hannover, Germany

Work experience

April 2014 to present: Laboratory of Molecular and Cellular Biology
University of Zurich, Vetsuisse Faculty, Institute of Veterinary Anatomy, Switzerland
PhD student

2014: Laboratory of Endocrinology
University of Veterinary Medicine Hannover, Germany
Student assistant

2007-2011: Eisenhauer Training, Goseriede 1-5, Hannover, Germany
Fitness instructor (shift supervisor and selling of fitness contracts)

June 2006-July 2007: Fitnessland GmbH, Engelbostler Damm 126, Hannover, Germany
Fitness instructor (including selling fitness contracts)

2005-2006: Zander Training, Goseriede 1-5, Hannover, Germany
Fitness instructor (including selling fitness contracts)

2005: Pegasus Pharma GmbH, Feodor-Lynenstrasse 5, Hannover, Germany
Temporary employee

2003-2004: Department of Hematology, Medical School Hannover, Germany
Civilian service

Language skills
German: native language
English: fluent

Publications

- *Uterine and placental distribution of selected ECM components in the dog*.
  Graubner FR, Boos A, Aslan S, Kücükaslan I, Kowalewski MP.
  Reproduction. 2018 May 1;155(5):403-421 doi: 10.1530/REP-17-0761. Epub 2018 Feb 9. PMID: 29439094

- *Uterine responses to early pre-attachment embryos in the domestic dog and comparisons with other domestic animal species*.
  Graubner FR, Gram A, Kautz E, Bauersachs S, Aslan S, Agaoglu AR, Boos A, Kowalewski MP.
  Biology of Reproduction 2017 Aug 1; 97(2):197-216. doi: 10.1093/biolre/iox063. PMID:28651344

- *Decidualization of the canine uterus: From early until late gestational in vivo morphological observations, and functional characterization of immortalized canine uterine stromal cell lines*.
  Graubner FR, Reichler IM, Rahman NA, Payan-Carreira R, Boos A, Kowalewski MP.
  Reproduction in domestic animals. 2017 Apr; 52 Suppl 2:137-147.
  doi: 10.1111/rda.12849. Epub 2016 Nov 15. PMID:27862405

- *The Dog: Nonconformist, Not Only in Maternal Recognition Signaling*.
  Kowalewski MP, Gram A, Kautz E, **Graubner FR**.
  Advances in anatomy, embryology, and cell biology. 2015; 216:215-37. doi: 10.1007/978-3-319-15856-3_11. PMID: 26450501

Acknowledgments

I would like to thank everyone who contributed to the present thesis, especially the following people:

Prof. Dr. Mariusz P. Kowalewski, my supervisor in the Institute of Veterinary Anatomy, University of Zurich. He accepted me into his laboratory although we had only briefly met during my practical year in the end of my veterinary studies. I am thankful that he gave me the opportunity to work with him. Prof. Kowalewski taught me a lot. Here I would like to mention his most important lessons to me about canine reproduction and the passion for research.

Prof. Dr. Susanne Ulbrich, from the Institute of Agricultural Sciences, Animal Physiology from the Swiss Federal Institute of Technology (ETH) Zurich, who was the co-supervisor of this study. Her great input during discussion sessions following presentations has always been appreciated.

Prof. Dr. Daniel Fuster, from the Inselspital, Bern University Hospital, who accepted being my mentor. His polite and rational character made it very pleasant to work with him.

The Graduate School Bern (GCB) and the PhD Expert Committee to accept me as a PhD student.

Prof. Dr. Alois Boos, the director of the Institute of Veterinary Anatomy, UZH, Zurich. I have belonged to his Institute since April 2014. He always gave me great feedback for improvement of papers, posters and presentations. I am grateful that he accepted me in his Institute so that I had the chance to experience both the daily life of a scientist and the fun of teaching veterinary anatomy to future veterinarians.

JProf. Dr. Marion Schmicke from the Clinic for Cattle, University of Veterinary Medicine, Hannover. I am grateful that she accepted me as an undergraduate student in her lab. With this she strongly supported my early scientific career.

Dr. Aykut Gram, who would always give great advice and feedback. We shared hotel rooms for every congress and got along very well. Tesekkür Abi.

Marta Nowak, Bettina Lanfranchi and Miguel Tavares Pereira, the PhD students with whom I shared an office. All of them are smart and very friendly. Thank you for sharing your thoughts about research and also your private life with me.

I would also like to thank everybody who is affiliated with the Institute of Veterinary Anatomy, for a great time (4 years) at work and in Zurich.

I would also like to thank special people outside of Science:
The biggest acknowledgment goes to my family that has supported me immensely. All of you are an important part of my life. We have maintained our contacts on a regular basis and our bond has never been better.

I am especially grateful to my grandmother (and grandfather who unfortunately passed away some time ago), because you two supported me throughout my studies and veterinary-technician education. Thank you so much.

My parents, who are still a very important part of my life and I am deeply grateful that I have these two at my side.

A special thanks goes to my friends at Irchel-building Y30:
Dr. Firat Durmaz, Eric Gahviler, Kevin Michaelsen, Andreas Rüttimann, Dr. Jakub Kubacki, Lilian Hegglin, Hanno Kappen and Konrad Harbeck.

Declaration of Originality

Declaration of Originality

Last name, first name: Graubner, Felix

Matriculation number: 14-714-430

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Zurich, 17.04.2018

Signature F. Graubner